Structural aspects on the metabolism of Glucocorticosteroids.

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Abstract

Topical glucocorticosteroids (GCS) are highly effective in the the local treatment of asthma, rhinitis and skin diseases. The metabolism of GCS occurs mainly in the liver and leads to a reduction of their biological activity. In order to achieve a high local therapeutic effect with low systemic side effects, agents with a high potency and a rapid liver biotransformation are desirable. The effects of structural alteration on the biotransformation rate of GCS in liver 9000 g supernatant fractions are described.

Insertion of a 1,2-double bond and a 16α-hydroxy group decrease the rate of biotransformation of GCS in human and rat liver. An enhancement on the rate of biotransformation in human liver is obtained upon substitution with a symmetric and a non-symmetric 16α,17α-acetal group or with 6α- and 9α-fluoro atoms. Fluorination in 6α- or 9α-position have only a marginal effect on the rate of biotransformation in rat liver preparations, thus differing from the results obtained with human liver. Furthermore, the GCS containing the symmetric 16α,17α-acetal substituent are slowly biotransformed in rat liver preparations. The significance of these observations is discussed.

Budesonide is a highly potent topical GCS. The non-symmetric 16α,17α-acetal substituent present in this drug increases the therapeutic activity and provides at the same time sites for metabolic inactivation in the liver. After intravenous administration to mouse, budesonide rapidly escapes the blood and is concentrated in various tissues, particulary in liver and kidney but also in lung and lymphatic tissues. Only low levels are found in the CNS.

Key words

Glucocorticosteroids - liver - metabolism - biotransformation - pharmacokinetics - structural aspects - tissue distribution

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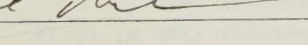
Signature 

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STRUCTURAL ASPECTS ON THE METABOLISM OF GLUCOCORTICOSTEROIDS.

By

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PAUL ANDERSSON

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APPENDIX (papers I-VI)

This thesis is based on the following publications which will be referred to in the text by their Roman numerals:

I Andersson,P., Brattsand,R., Edsbäcker,S., Källström,L. and Ryrfeldt,Å.: Biotransformation rate (in vitro) and systemic potency (in vivo) of the topical glucocorticoid budesonide in male and female rats. **J. Steroid Biochem. 17, 1982, 703-706.**

II Andersson,P., Edsbäcker,S., Ryrfeldt,Å. and von Bahr,C.: In vitro biotransformation of glucocorticoids in liver and skin homogenate fraction from man, rat and hairless mouse. **J. Steroid Biochem. 16, 1982, 787-795.**

III Andersson,P., Lihné,M., Thalén,A. and Ryrfeldt,Å: Effect of structural alterations on the biotransformation rate of glucocorticosteroids in rat and human liver. **Xenobiotica, in press.**

IV Andersson,P. and Ryrfeldt,Å: Biotransformation of the topical glucocorticoids budesonide and beclomethasone 17α,21-dipropionate in human liver and lung homogenate. **J. Pharm. Pharmacol. 36, 1984, 763-765.**

V Edsbäcker,S., Andersson,P., Lindberg,C., Ryrfeldt,Å. and Thalén,A.: Metabolic acetal splitting of budesonide - a novel inactivation pathway for topical glucocorticoids. **Submitted for publication.**

VI Andersson,P., Appelgren,L-E. and Ryrfeldt,Å.: Tissue distribution and fate of budesonide in the mouse. **Acta Pharm. Toxicol. 59, 1986, in press.**

NOMENCLATURE

The glucocorticoids discussed in this thesis have been named according to their structural divergence from either prednisolone, desonide or budesonide. Most of these glucocorticoids have also well known generic names, which in some cases appear in the text. For a complete understanding, the reader is referred to the list given below, in which both structural and generic names are given. The numbering of the steroid skeleton is shown in Fig.1.

Structural name	Generic name	Fig.
Prednisolone	Prednisolone	2
1,2-Dihydroprednisolone	Hydrocortisone	2
16α-Hydroxyprednisolone	Prednacinolone	3
9α-Fluoro,16α-hydroxyprednisolone	Triamcinolone	3
6α,9α-Difluoro,16α-hydroxyprednisolone	Fluocinolone	3
16β-Methylprednisolone 17α-propionate	-	7
16β-Methylprednisolone 17α-valerate	-	7
9α-Fluoro,16β-methylprednisolone - 17α-propionate	Betamethasone 17α-propionate	7
9α-Fluoro,16β-methylprednisolone - 17α-valerate	Betamethasone 17α-valerate	7
9α-Chloro,16β-methylprednisolone - 17α-propionate	Beclomethasone 17α-propionate	7
9α-Chloro,16β-methylprednisolone - 17α,21-dipropionate	Beclomethasone 17α,21-dipropionate	6
Desonide	Desonide	4
9α-Fluorodesonide	Triamcinolone acetonide	4
6α-Fluorodesonide	Flunisolide	4
6α,9α-Difluorodesonide	Fluocinolone acetonide	4
Budesonide*	Budesonide	5
1,2-Dihydrobudesonide*	-	5
9α-Fluorobudesonide*	-	5
6α,9α-Difluorobudesonide*	-	5

* = these glucocorticoids exist as two epimers with either 22R or 22S configuration.

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INTRODUCTION

Introductory remarks

The effect of structural alterations on the metabolism of glucocorticosteroids (GCS) was a very important area for research during the period 1955-1965. Then several potent GCS were synthetized for the first time, and it was generally believed that the increased biological activity observed with these GCS, was due to a slow rate of biotransformation (inactivation). In these early investigations, the metabolism rate was measured by different color reactions like the Porter-Silber, blue tetrazolium and Gornall reaction, but also by uv-absorption methods and chromatography on paper strips. In the last 20 years, several other even more active GCS have been synthetized. However, the research on the metabolism of these GCS has been merely of documentative character. Little attention has been focused on comparative studies and to what extent species differences occur. Today the opinion is that the biological activity of drugs is regulated by their potency, intrinsic activity and concentration at the site of action. This relationship is shown in the following equation:

$$\text{biological activity} = M_p \cdot M_{i.a.} \cdot [M]$$

M_p = potency of active molecule (receptor affinity)

$M_{i.a.}$ = maximal activity produced by the drug-receptor complex (intrinsic activity)

[M] = concentration of active molecule at the site of action

[M] is determined by kinetic factors like absorption, distribution, metabolism and excretion. This thesis mainly deals with structural aspects on the metabolic rates of GCS, although some attention will also be given to the tissue distribution.

Biological effects of glucocorticosteroids

The corticosteroids have a very broad spectrum of action, but there are two major effects: they influence electrolyte balance (mineralcorticoid action) and carbohydrate metabolism (glucocorticoid action). The endogenous GCS in man, hydrocortisone, has a pronounced action on carbohydrate metabolism but only a slight action on electrolyte balance. Conversely, the glucocorticoid action of the endogenous mineralcorticosteroid aldosteron is essentially zero. The synthesis and secretion of hydrocortisone is regulated via a feed-back mechanism, known as the hypothalamus-pituitary-adrenal-axis (HPA-axis) (review Haynes and Murad, 1980). In addition to effects on carbohydrate metabolism, GCS also possess antiinflammatory, antiallergic and immunosuppressive action which are thought to be their most important therapeutic properties. Today, GCS are the most effective drugs known for treatment of eg., asthma, rhinitis, rheumatoid arthritis and psoriasis. The mechanisms of action are still far from being completely understood. However, after passive diffusion into the cells, the GCS bind to specific receptor proteins. After translocation of the steroid-receptor complex into the cell nucleus, synthesis of specific messenger RNA species will be induced, and these code for specific proteins. One such protein is lipocortin which is thought to be intimately linked to the antiinflammatory action of GCS (Flower, 1986). Lipocortin blocks the enzyme phospholipase A_2 that is involved in the formation and liberation of inflammatory mediators such as prostaglandins and leukotrienes.

Structure-activity relationship-historical background

The numbering of the steroid skeleton is shown in Fig.1.

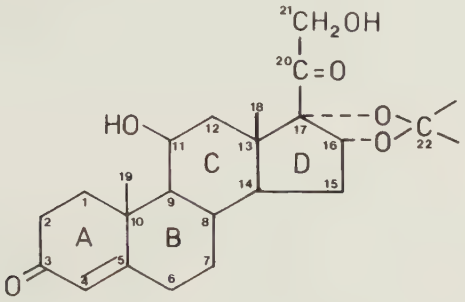


Fig.1.

Systemic glucocorticoids

The term "systemic GCS" refers to GCS which are used for oral systemic treatment of inflammatory diseases such as rheumatoid arthritis, lupus erythematosus and allergic diseases like asthma. Usually, the systemic treatment of these diseases are associated with various side effects like those observed in Cushings syndrome, eg., adrenal atrophy, increase in insulin requirement, osteoporosis, mental alertness, increased or decreased appetite. Hydrocortisone (Fig.2), which is the main endogenous GCS in man, was isolated in 1942 by Reichstein and Shoppee (1943) and first synthetized in 1950 by Wendler et al. (1950).

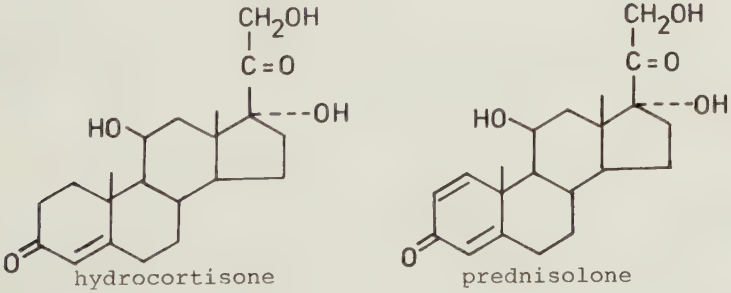


Fig.2.

The discovery of the beneficial effects of hydrocortisone in the treatment of rheumatoid arthritis (Bowland and Headly, 1952; Polly and Mason, 1950) led to an extensive research on how to improve the effects by modifying the steroid structure. One of the first successful modifications made was the insertion of the 1,2-double bond giving prednisolone (Fig.2). This modification resulted in a 4-fold increase in glucocorticoid activity without a proportional increase in mineralcorticoid activity (Herzog et al., 1955).

Another way of improving the biological activity of hydrocortisone was the substitution with a fluoro atom at position 9α (Fried and Sabo, 1954). The substitution resulted in a 10-fold increase in antirheumatoid activity, but, unfortunately, the mineralcorticoid activity increased to an even greater degree (Bowland, 1955). Although insertion of the 1,2-double bond further increased the antirheumatoid activity (Hirschmann et al., 1955), it did not decrease the mineralcorticoid activity introduced by the 9α-fluoro atom (Black et al., 1956). A major break-through came when Freyberg and coworkers (1958) tested the biological activity of triamcinolone (Fig.3, 9α-fluoro, 16α-hydroxyprednisolone). Although the presence of the 16α-hydroxy group decreased the antiinflammatory activity, triamcinolone still retained enough activity to be approximately equivalent to prednisolone when given systemically. More important, however, was the fact that the sodium-retaining characteristics of the 9α-fluoro atom were overcome. The synthesis of the difluorinated compound fluocinolone (Fig.3, 6α,9α-difluoro, 16α-hydroxy-prednisolone) resulted in a 7-fold increase of the glucocorticoid activity compared to triamcinolone with no retention of sodium (Mills et al., 1960). The non-fluorinated analog prednacinolone (Fig.3, 16α-hydroxyprednisolone) has a very low glucocorticoid activity (Bowland, 1961; Dahlberg et al.,1984).

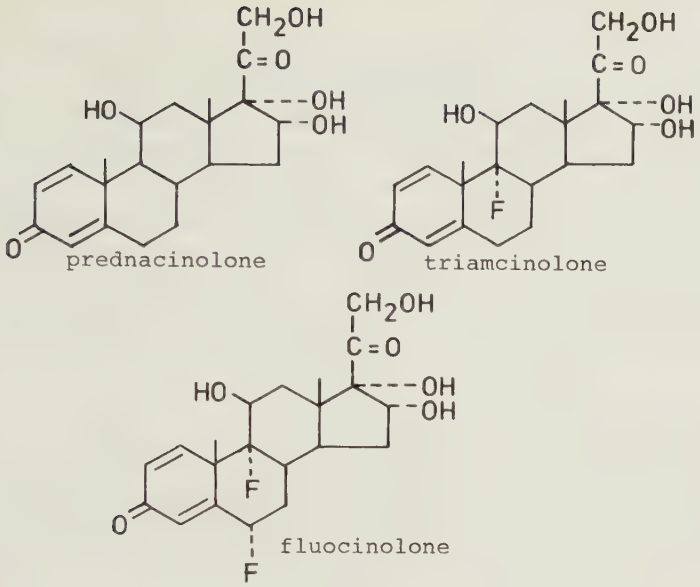


Fig.3.

Topical glucocorticoids

The term "topical GCS" refers to highly potent GCS which are suitable for local treatment of asthma, rhinitis, psoriasis and probably also ulcerative colitis. Most of the topical GCS have glucocorticoid potencies more than 100-fold that of hydrocortisone. Partly this feature makes them suitable for local treatment in doses that only minimally affect important systemical functions as the HPA-axis. Topical GCS can be divided into two structurally different groups, namely the acetal and ester GCS.

Acetal GCS: These GCS have been synthetized by masking the 16α, 17α-hydroxy groups with either a symmetric or a non-symmetric acetal.

The structures of the symmetric acetal GCS, differing only in 6α- and 9α-fluorination, are given in Fig.4. These drugs were synthetized in the late fifties and early sixties (Fried et al., 1958; Mills et al., 1960) and soon proved to be effective in the local treatment of skin diseases (Robinson, 1961; Rostenberg,

1961). Today desonide, triamcinolone acetonide (9α-fluorodesonide) and fluocinolone acetonide (6α,9α-difluorodesonide) are marketed for topical use on the skin. Inhaled flunisolide (6α-fluorodesonide) was reported by Lowell et al. (1976) to be effective in treatment of asthma. The doses required for therapeutic effect was not associated with significant systemic side effects (Chaplin et al., 1980_b). Flunisolide is now marketed as an aerosol for treatment of asthma and as a nasal spray for treatment of allergic rhinitis (Kwaselow et al., 1985).

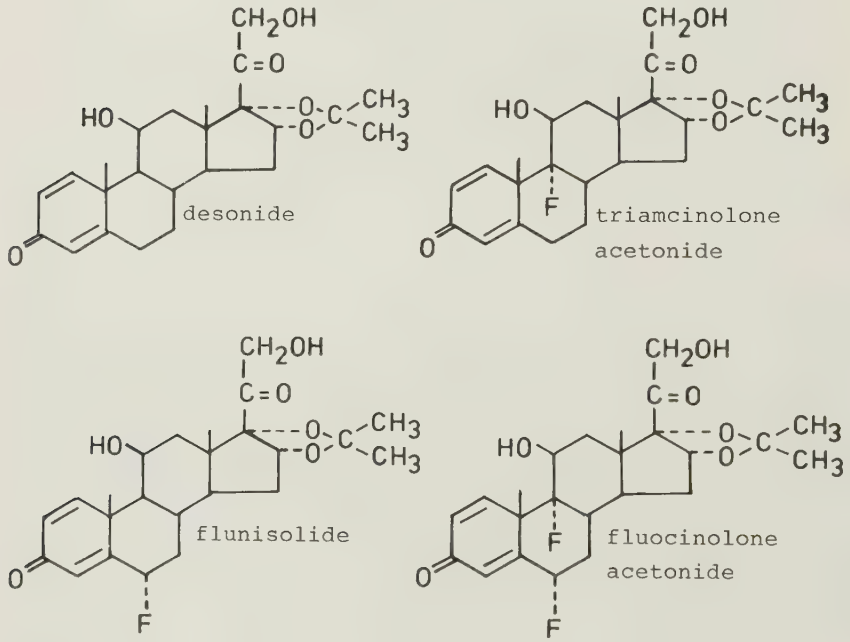
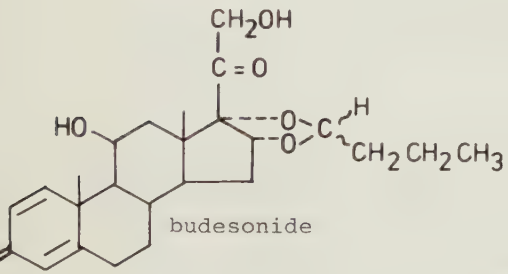


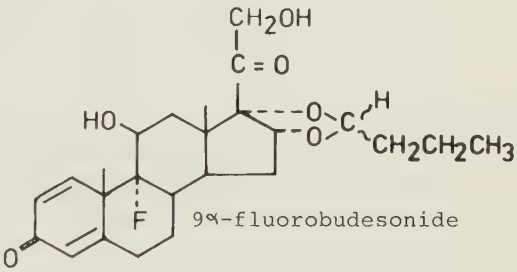
Fig.4.

The synthesis of the non-symmetric acetal GCS was described by Thalén and Brattsand (1979). Budesonide (Fig.5) being a mixure of two epimers with 22R and 22S configuration had a local anti-inflammatory activity comparable to that of fluocinolone acetonide, but a systemic glucocorticoid activity 4-7 times

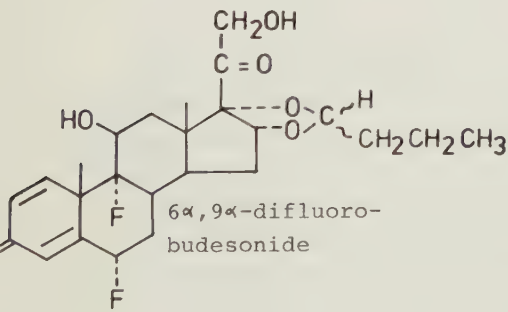
lower, as measured in rats (Thalén and Brattsand, 1979). The relationship between topical and systemical activities of the fluorinated analogs 9α-fluorobudesonide and 6α,9α-difluoro-budesonide was less favourable than that of budesonide (Brattsand et al., 1982_a). The local antiinflammatory activity of 1,2-dihydrobudesonide in rat was equal to that of budesonide. Interestingly, however, its activity to involute thymus after subcutaneous administration was 10 times lower (Thalén et al., 1984). Among these GCS, budesonide is therapeutically used for topical treatment of asthma (Johansson et al., 1982), intranasal treatment of rhinitis (Pipkorn et al., 1980) and in various formulations for treatment of skin diseases (Agrup et al., 1981) without causing any significant systemic distubances in the therapeutic dose range (review Clissold and Heel, 1984). At present, work is in progress to estimate its relative efficacy in the treatment of ulcerative colitis.



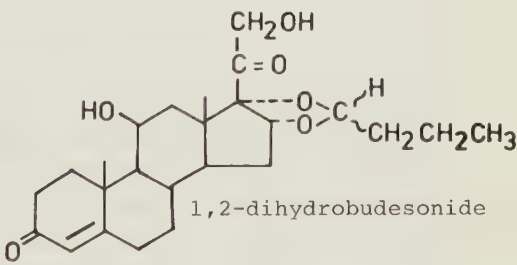
budesonide



9α-fluorobudesonide



6α,9α-difluoro-budesonide



1,2-dihydrobudesonide

Fig.5.

Ester GCS: The introduction of a 16α- or a 16β-methyl group in 9α-fluoroprednisolone was found to increase the antiinflammatory activity over the parent molecule and, at the same time, to abolish the sodium-retaining characteristics introduced by the 9α-fluoro atom (Silber, 1959; Taub et al., 1960). At that time, the 16α- or 16β-methylprednisolone derivatives served as starting material for synthetizing a new class of GCS, namely the 17α- and/or 21-ester GCS.

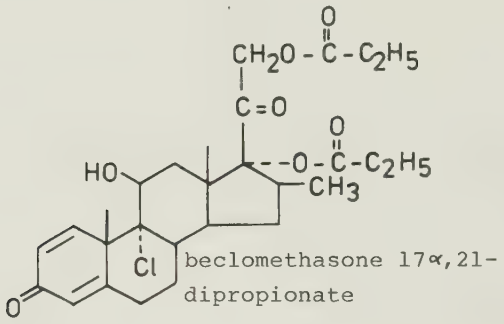
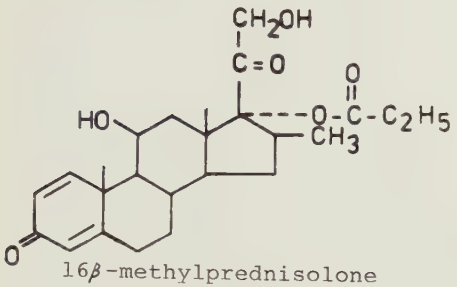


Fig.6.

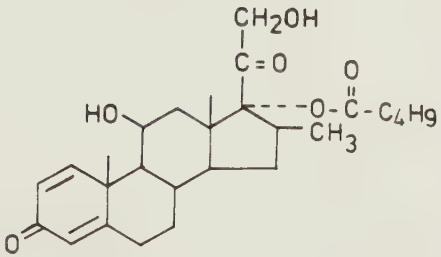
The therapeutic efficacy of beclomethasone 17α,21-dipropionate (Fig.6) in the topical treatment of asthma and rhinitis is well established today. Its topical activity is around half that of budesonide (Johansson et al., 1982), and the treatment is not associated with significant side effects (review Brogden et al., 1984).

In Fig.7, the structures of some 17α-monoester GCS are shown. Beclomethasone 17α-propionate (9α-chloro, 16β-methylprednisolone 17α-propionate) results from hydrolysis of the 21-ester group in beclomethasone 17α,21-dipropionate, and has a topical activity of around 3/4 of the latter drug (Harris, 1975). Betamethasone 17α-valerate (9α-fluoro, 16β-methylprednisolone 17α-valerate) is used for treatment of skin disorders, but is also therapeutically active in the topical treatment of asthma (Mc Allen et al.,1974), and rhinitis (Brostoff and Czainy, 1968; 1969), and in the oral treatment of ulcerative colitis and Crohns disease (Friedman et

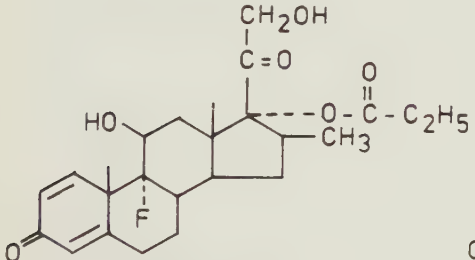
al., 1967; Gill et al., 1965) without demonstrating any significant adverse effects.



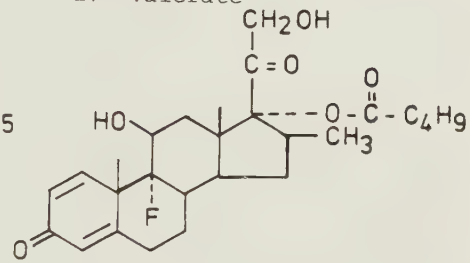
16β-methylprednisolone
17α-propionate



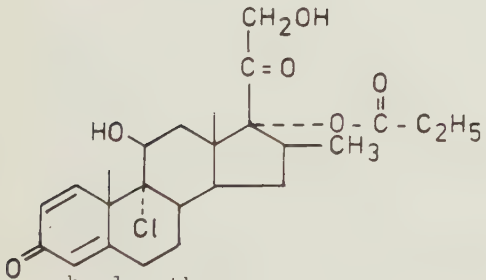
16β-methylprednisolone
17α-valerate



betamethasone
17α-propionate



betamethasone
17α-valerate



beclomethasone
17α-propionate

Fig.7.

Topical treatment - the key role of the liver

As mentioned earlier, oral systemic treatment with GCS is unfortunately associated with various systemic side effects. This led pharmacologists to consider that a reduction of systemic effects with the retention of therapeutic action might be achieved by application of the drug directly to the target organ (lung, skin) in smaller amounts than would generally be administered systemically. Concerning topical treatment of skin diseases, these considerations proved to be right. However, early studies on topical treatment of asthma with aerosolized hydrocortisone and dexamethasone phosphate were discouraging (Foulds et al., 1955; Dennis and Itkin, 1964). Hydrocortisone was practically inactive and dexamethasone phosphate, although therapeutically active, produced unwanted systemic effects. It was not until beclomethasone 17α,21-dipropionate and betamethasone 17α-valerate became available that topical treatment of asthma was feasible. At that time, it was realized that a patient using a drug by aerosol could be regarded as having two doses, an inhaled dose, being that portion (~10%) which enters the lung and an oral dose, being the much larger portion (~90%) which is swallowed (Newman et al., 1981; Davies, 1982). If the inhaled fraction is sufficiently large for therapeutic effect, it would be of advantage if the swallowed fraction was inactivated before reaching the systemic circulation. Since orally administered drugs almost entirely pass through the liver via the portal circulation before entering the general circulation, hepatic drug metabolizing enzymes may have an important role in minimizing systemic side effects of aerosolized GCS. Accordingly, the highly favourable ratio between topical and systemical effects seen with the GCS budesonide is thought, at least in part, to be due to its efficient inactivation in the liver (Ryrfeldt et al., 1982).

AIMS OF THE STUDIES

The aims of the present studies have been to investigate:

* The effect of liver metabolism on the systemical activity of the topical glucocorticoid budesonide in rat (I).

* The effect of structural alterations on the liver metabolic rate of glucocorticoids in rat and man (II, III, IV).

* The mechanism behind the unusual inactivation pathway of budesonide - the acetal side chain cleavage (V).

* The tissue distribution of budesonide - a factor possibly contributing to the favourable ratio between topical and systemical activity seen with this drug (VI).

Only a short survey of the methods used will be given. For a complete and detailed description, the reader is referred to the original papers.

In vitro studies

These studies were performed to compare the biotransformation rate of structurally related GCS and to elucidate the mechanism behind the metabolic acetal splitting of budesonide.

Liver and lung specimens

Rat and mouse livers were perfused in situ and removed after decapitation of the animals. Skin samples were taken from the back of shaved animals. Human liver samples were supplied from the liver bank at Huddinge hospital by Dr. von Bahr. They were taken from cadaveric renal transplant donors shortly after circulatory arrest and immediately frozen at -196°C. They were then stored at -80°C. Storage under these conditions preserve most enzyme activities for at least 6 month (von Bahr et al., 1980). Human lung samples without any visible pathological changes were taken during lobectomy at Lund hospital. The samples were kept on ice for about 1 hr and then frozen at -80°C.

Incubation

All GCS (either tritium labelled or non-labelled) were incubated with liver 9000 g supernatant fractions at 37°C in presence of NADPH, $MgCl_2$ and O_2-athmosphere (95% O_2, 5% CO_2). Some incubations were also performed with lung 1000 g and skin 9000 g supernatant fractions. The concentration of tritium labelled GCS were 1.0 or 0.1 umol/l and in the case of non-radiolabelled GCS 5.0 umol/l. Aliquots for analysis were taken several times after start of each incubation.

Purification procedure

Prior to analysis, the remaining unmetabolized GCS were purified on Sep pak, C-18 cartridges or by shaking with dichloromethane. The recovery, as determined from radioactivity measurements was >85%.

Liquid chromatography

Separation of unchanged compound from metabolites was performed on Nucleosil C_{18}, 5 um particles, using either isocratic or gradient elution with ethanol/water mixtures. The elution of non-labelled GCS was recorded by a uv-detector (254 nm). In experiments with tritium labelled compounds, fractions were collected for radioactivity measurments. In some experiments, an on line radioactivity detector was used.

Quantification

Radioactivity was measured with a liquid scintillation detector, equipped with external standard for determination of counting efficiency. During chromatography, the area under the uv-peaks (AUP) was calculated by a microprocessor. Unmetabolized GCS was quantified by dividing either the radioactivity of tritium labelled compound or the AUP of non-labelled compound with the AUP of an internal standard. The concentration was then obtained from standard curves.

Mass spectrometry

Chemical ionization (methane) mass spectra were obtained using a moving belt LC-MS interface. Stable isotopes (^{2}H-budesonide and $^{18}O_2$) were used to facilitate interpretation of mass spectral data.

In vivo studies

These studies were performed to compare the systemic activity of budesonide in male and female rats and to study the tissue distribution and pharmacokinetics of budesonide in mice.

Systemic activity

Budesonide was given as a suspension, either orally or subcutaneously, to male and female rats. Reduction of thymus weight, being a sensitive parameter for systemic glucocorticoid action, was determined.

Pharmacokinetics

The pharmacokinetics of tritiated budesonide was studied after intravenous administration to mice. Separation of budesonide in blood from metabolites was performed by liquid chromatography on Nucleosil NO_2, 5 um particles, using a step gradient elution with dichloromethane/isopropanol/water mixtures. Quantification was performed as described above.

Tissue distribution

Whole body autoradiography of mice injected intravenously with tritiated budesonide was performed according to Ullberg (1954; 1977). Chromatography of tissue radioactivity was made on Nucleosil C18, 5 um particles, using a step gradient elution with ethanol/water mixtures.

Calculations

During incubation with liver preparations, the GCS disappeared in a concentration dependent manner suggesting first order kinetics. The in vitro half life was calculated from $\ln 2/\beta$, where β is the slope of the regression line of the log-linear concentration-time curve.

After intravenous administration of budesonide to mice, kinetic parameters were calculated from the mean blood concentration time curve of unchanged budesonide. The terminal elimination rate constant (β) was obtained by linear regression of the last log-linear portion of the curve. The terminal elimination half life was then calculated as $\ln 2/\beta$. The distribution rate constant (α) was calculated by curve stripping and linear regession of the first 10 min of the resulting curve. The distribution half life was then calculated as $\ln 2/\alpha$. The area under the blood concentration time curve (AUC) up to 240 min was determined by the trapetzoid rule. The remaining area was calculated as Cb(240 min)/β. Total blood clearance was calculated as Dose/AUC_{tot}. The apperant volume of distribution (V_{β}) was calculated as clearance/β.

Relationship between liver metabolism and systemic activity as exemplified with budesonide (I)

It is well known that sex differences in the biotransformation of drugs including GCS occur in the rat. Briefly, male rats have a higher capacity to biotransform drugs than female rats in vivo as well as in vitro. Thus, the rat may serve as a model for investigations concerning the relationship between biotransformation and biological activity of GCS.

Tritium labelled budesonide was incubated (1 umol/l) with male and female rat liver 9000 g supernatant fraction. The metabolic disappearance curves of unchanged budesonide are shown in Fig.8. Budesonide disappeared about 4 times more rapidly in livers from male than from female rats.

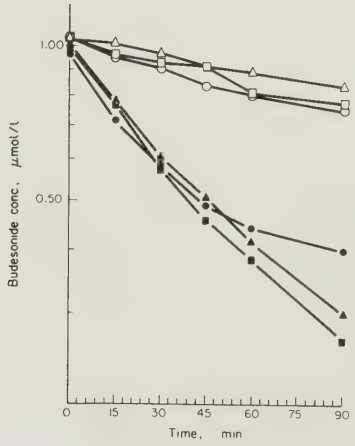


Fig.8. Metabolic disappearance of budesonide during incubation with male (filled symbols) and female (open symbols) rat liver 9000 g supernatant fraction.
○: experiment 1, Δ: experiment 2, □:experiment 3.

To investigate the systemic activity of budesonide, the drug was administered orally and subcutaneously. Reduction of thymus weight was determined, being one of the most sensitive parameters for systemic glucocorticoid activity. The ED_{50} values, defined as the dose reducing thymus weight to 50% of that in the control group, are given in Table 1.

Table 1. Relative activity of budesonide to involute thymus weight in male and female rats. ( )=95% confidence limits.

	ED_{50} (mg/kg)	
	Oral	Subcutaneous
Male rats	2.54 (1.97-3.40)	0.33 (0.27-0.40)
Female rats	0.43 (0.32-0.67)	0.19 (0.14-0.27)

It is evident that budesonide was about 6 times more active in female than in male rats after oral administration. After subcutaneous administration, budesonide was only about twice as active in female rats. These results suggest that the liver is capable of reducing the systemic activity of budesonide, especially after oral administration.

Biotransformation rates of GCS in liver and skin from different species - a comparison between hydrocortisone, triamcinolone-acetonide and budesonide (II)

Tritiated hydrocortisone, triamcinolone acetonide and budesonide were incubated with liver and skin 9000 g supernatant fractions from man, rat and hairless mouse. The incubation concentrations were 1.0 and 0.1 umol/l. The metabolic disappearance rates (expressed as $t_{1/2}$-values) in liver are given in Table 2.

Budesonide is a mixure of two epimers with 22R and 22S configuration. The $t_{1/2}$-values for these epimers during incubation of budesonide with liver are shown in Table 3.

The results can be summarized as follows:
1. The rank order of biotransformation rates in human and mouse liver was budesonide>triamcinolone acetonide>hydrocortisone.

2. The rank order of biotransformation rates in rat liver was hydrocortisone>budesonide>triamcinolone acetonide, thus differing from that in human and mouse liver.

3. The 22R-epimer of budesonide was more rapidly biotransformed than the 22S-epimer, especially in mouse liver.

4. No biotransformation at all was found during incubation of the GCS with skin preparations.

Since the GCS studied structurally differed from each other in more than one respect, it was not possible to estimate the relative influence of the 1,2-double bond, the 9α-fluoro atom or the two kinds of 16α,17α-acetal substituents. Yet, the results indicated species differences concerning the influence of these structural elements. Further studies were therefore performed.

Table 2. The metabolic disappearance rates of GCS during 30 min of incubation with liver 9000 g supernatant fraction. Range values are given. Number of incubations = 5(human liver), 1-2(rat liver), 2(mouse liver).

		$t_{1/2}$ (min)		
		Human	Rat	Mouse
Compounds	umol/l	(M+F)	(M)	(M)
Hydrocortisone	1.0	41-67	14-21	140-165
	0.1	40-42	17	82-113
Triamcinolone-acetonide	1.0	13-67	161-186	32-34
	0.1	17-68	196	21-24
Budesonide	1.0	7-23	28-38	20
	0.1	8-22	33	17-22

M = male, F = female

Table 3. The metabolic disappearance rates of the 22R- and 22S-epimers during 30 min of incubation of budesonide with liver 9000 g supernatant fraction. Range values are given. Number of incubations = 5(human liver), 1-2(rat liver), 2(mouse liver).

		$t_{1/2}$ (min)		
		Human	Rat	Mouse
Compounds	umol/l	(M+F)	(M)	(M)
22R-Epimer	1.0	6-14	23-29	8
	0.1	6-16	25	7-8
22S-Epimer	1.0	8-38	36-48	37-38
	0.1	9-31	44	26-36

M = male, F = female

Biotransformation rates of GCS in liver - the influence of 1,2-hydrogenation, 16α-hydroxylation, 16α,17α-acetal substitution, and 6α,9α-fluorination (III)

A number of non-radiolabelled steroids were incubated at 5 umol/l with rat and human liver 9000 g supernatant fraction. The results are summarized in Table 4.

The following comments on the results can be made:

1. Hydrogenation of the 1,2-double bond in prednisolone and in the epimers of budesonide enhanced the biotransformation rate about 8-fold in rat liver but only about 2-fold in human liver.

2. Steroid nucleus fluorination in 6α- and/or 9α-positions, enhanced the biotransformation rate of the acetal GCS severalfold in human liver but in rat liver, fluorination was found only to marginally affect the biotransformation.

3. Insertion in prednisolone of a 16α-hydroxy group reduced the biotransformation rate at least 2-fold in both rat and human liver.

4. Masking of the 16α,17α-hydroxy groups with the non-symmetric acetal (present in eg. budesonide), enhanced the biotransformation rate severalfold, particulary in human liver. The 22R-epimers of these GCS were more rapidly biotransformed than the 22S-epimers (about 1.5 - 2 times).

5. Masking of the 16α,17α-hydroxy groups with a symmetric acetal (present in eg. desonide) also efficiently enhanced the biotransformation rate in human liver. In rat liver, however, the enhancing effect was not very pronounced. In general, the symmetric acetal GCS were poorly biotransformed in rat liver.

6. A comparison of the two types of acetal GCS containing the same number of fluorine atoms revealed that the non-symmetric acetal GCS were more rapidly biotransformed than the symmetric acetal ones.

Table 4. The metabolic disappearance rates of structurally related GCS during incubation with liver 9000 g supernatant fraction. Generic names of the GCS are given in the nomenclature list at the beginning of this thesis.

	$t_{1/2}$ (min)	
	Rat liver[a]	Human liver[b]
Systemical GCS		
Prednisolone	41.4	83.7
1,2-Dihydroprednisolone	4.9, 5.0*	37.2, 37.3*
16α-Hydroxyprednisolone	85.4	n.b.d.
9α-Fluoro,16α-hydroxyprednisolone	214	305
6α,9α-Difluoro,16α-hydroxyprednisolone	299	242
Topical GCS		
Desonide	67.0	52.5
9α-Fluorodesonide	117	16.2
6α-Fluorodesonide	123	5.8
6α,9α-Difluorodesonide	118	2.7
Budesonide (22R)	13.6, 18.0*	5.3, 6.1*
1,2-Dihydrobudesonide (22R)	2.0	3.3
9α-Fluorobudesonide (22R)	19.4	1.9
6α,9α-Difluorobudesonide (22R)	15.7	<1.0
Budesonide (22S)	29.7, 30.3*	10.8, 11.2*
1,2-Dihydrobudesonide (22S)	3.6	6.8
9α-Fluorobudesonide (22S)	28.0	3.9
6α,9α-Difluorobudesonide	37.2	1.6

n.b.d. = no biotransformation detected.
a = pooled livers from seven male individuals.
b = pooled livers from six individuals.
* = duplicate incubations.

Liver and lung metabolism of GCS - a comparison between budesonide and beclomethasone 17α,21-dipropionate and related GCS-esters (IV)

In this paper, the metabolism of budesonide was compared with that of beclomethasone 17α,21-dipropionate, a drug representing the ester type of topical GCS. The tritiated drugs were incubated (0.1 umol/l) with human liver 9000 g supernatant fractions from 4 individuals and with pooled human lung 1000 g supernatant fraction from 3 individuals.

On incubation with liver preparations, beclomethasone 17α,21-dipropionate instantaneously hydrolysed to beclomethasone 17α-propionate, a compound having similar biological activity as the parent compound. The rate of further biotransformation of beclomethasone 17α-propionate in comparison with budesonide is shown in Table 5.

Table 5. The metabolic disappearance rate of budesonide and beclomethasone 17α-propionate during incubation with 9000 g supernatant fractions from 4 human livers.

	$t_{1/2}$ (min)			
Liver	1	2	3	4
Budesonide	8	16	13	22
Beclomethasone 17α-propionate	18	61	42	53

Budesonide was 2-4 times more rapidly biotransformed than beclomethasone 17α-propionate. During the incubation of the latter compound, no beclomethasone could be detected, as would be the case if hydrolysis was an important metabolic route for this compound in the liver.

During incubation with human lung preparation, beclomethasone 17α,21-dipropionate was rapidly hydrolysed to beclomethasone 17α-propionate and then further to beclomethasone at a much slower rate (Fig.9). Budesonide was not biotransformed by the lung preparation.

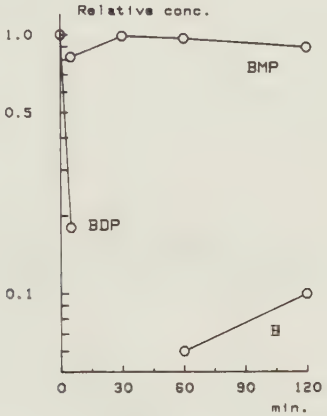


Fig.9. The metabolic disappearance of beclomethasone-17α,21-dipropionate (BDP) and the subsequent formation of beclomethasone 17α-propionate (BMP) and beclomethasone (B) upon incubation of BDP with human lung 1000 g supernatant fraction.

Some non-radiolabelled 17α-propionate and 17α-valerate esters were incubated (5 umol/l) with pooled human liver 9000 g supernatant fractions from 6 individuals.

The results (Table 6) show that the 17α-valerate compounds were more rapidly biotransformed than the 17α-propionate ones. Fluorination in the 9α-position enhanced their rate of biotransformation about 2-fold.

Table 6. The metabolic disappearance rate of some 17α-ester GCS upon incubation with human liver 9000 g supernatant fraction (unpublished results).

	$t_{1/2}$ (min)	
	17α-Propionate	17α-Valerate
16β-Methylprednisolone	109	12.5
9α-Fluoro,16β-methylprednisolone	62	5.9

Study with budesonide - metabolic acetal side chain splitting (V)

In liver, budesonide is rapidly biotransformed to 16α-hydroxy-prednisolone by an acetal side chain splitting mechanism involving only the 22R-epimer. This route of biotransformation also occurs with the 22R-epimers of 1,2-dihydrobudesonide, 9α-fluorobudesonide and 6α,9α-difluorobudesonide, but not with the GCS containing the symmetric 16α,17α-acetal substituent. Since this highly efficient metabolic pathway may explaine the rapid biotransformation observed with budesonide and its analogs, experiments were undertaken to examine this route in detail.

When esterase inhibitors were added together with budesonide to liver 9000 g supernatant fraction, no 16α-hydroxyprednisolone could be detected. Instead a new compound, identified as 16α-butyryloxyprednisolone (prednacinolone 16α-butyrate) was formed. Experiments showed that atmospheric oxygen was incorporated at the 22-carbon of budesonide by microsomal monooxygenases. The resulting hypothetical product is probably chemically labile and will rearrange to the energetically more favourable 16α-butyryloxyprednisolone. In the presence of liver esterase activity, this compound was instantaneously hydrolysed to 16α-hydroxyprednisolone and butyric acid (Fig.10).

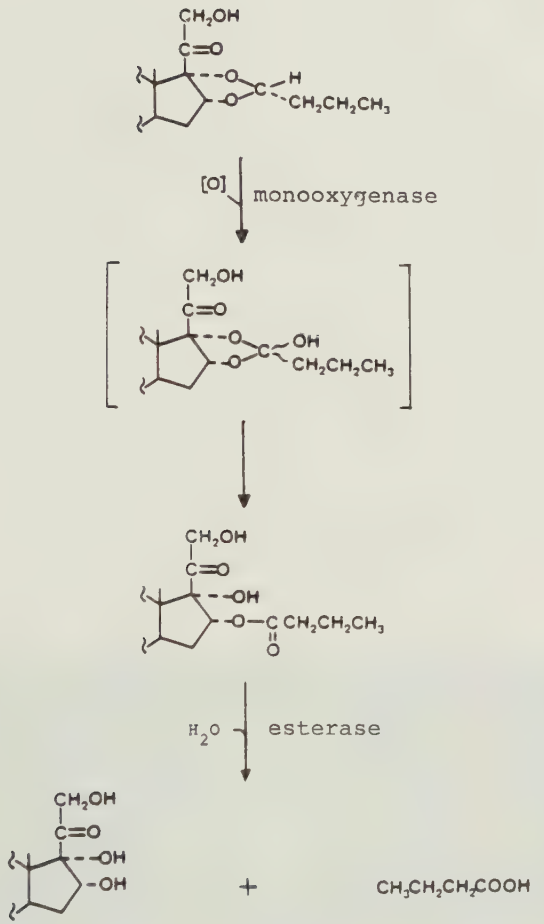


Fig.10. Suggested pathway for the 16α,17α-acetal splitting of the 22R-epimer of budesonide.

Study with budesonide - tissue distribution and fate in mice (VI)

Previous papers in this thesis have focused on liver biotransformation of GCS and the effect of structural alterations. Although important, biotransformation is only one of several kinetic parameters affecting the pharmacodynamics of GCS. Other kinetic parameters of importance are absorption, protein binding, distribution and excretion. Recently, some interest have been focused on the tissue distribution as a possible contributing factor to the favourable ratio between therapeutic effects and side effects seen with topical GCS. In the present study, the tissue distribution and the fate of tritiated budesonide were examined after intravenous administration to mouse.

Briefly, whole body autoradiography showed high amounts of radioactivity particularly in liver and kidney but also in lung and lymphatic tissue (Fig.11). Low levels were seen in blood and in CNS, with the exception of some activity in the cerebellum and a distinct but rapidly fading uptake of radioactivity in the pituitary.

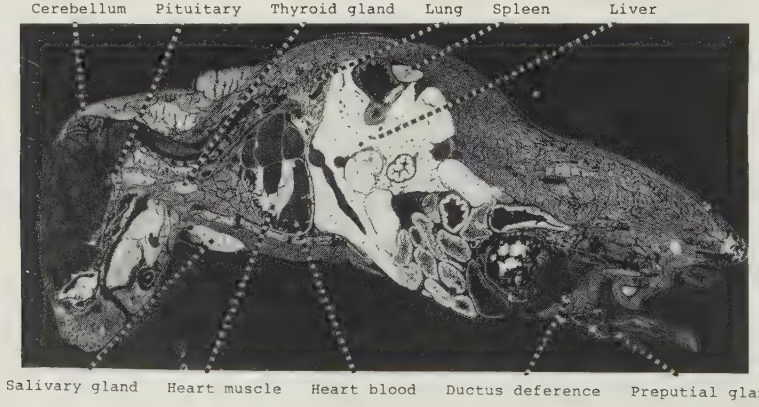


Fig.11. Autoradiogram of a male mouse 5 min after intravenous injection of tritiated budesonide. White areas indicate strong accumulation of tritium.

In a separate experiment, the kinetics was studied in more detail. The concentration time curves in blood of total radioactivity and unchanged budesonide are shown in Fig.12.

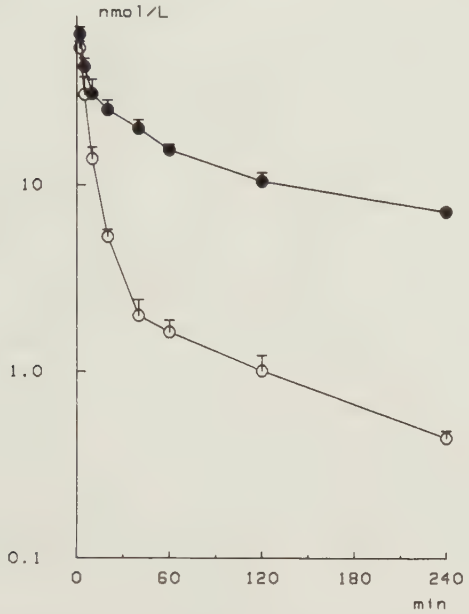


Fig.12. Concentration in blood of total radioactivity (●) and unchanged budesonide (○) after intravenous injection of tritiated budesonide (117 nmol/kg) to male mice. Mean values ± S.D. are given (n=5).

After administration, budesonide rapidly escaped the blood ($t_{1/2\alpha}$ = 3.7 min). Distribution equilibrium was attained after 40 min. The appearant volume of distribution (V_β) was calculated to 20.3 l/kg. From the terminal exponential phase (distinct from 40 min), the elimination half life was calculated to 1.55 hrs. Blood clearance was 9.04 l/hr/kg. These data show an extensive distribution of budesonide into tissues. Despite this, the drug was rapidly eliminated from the body, probably predominantly by hepatic metabolism. The tissue/blood concentration ratios of

radioactivity attributable to unchanged budesonide (Table 7) show a high tissue affinity of the drug.

Table 7. Tissue/blood concentration ratio of radioactivity attributable to unchanged budesonide after intravenous administration to male mice. Samples from 5 individuals were pooled before analyses.

Time (min)	Liver	Kidney	Lung	Spleen	Brain
5	5.0	12.5	4.2	1.8	0.9
20	9.7	25.5	11.5	9.3	2.8
60	8.9	22.3	23.9	22.0	4.8
240	13.9	23.8	53.3	34.2	3.7

Enzymes involved in the metabolism of GCS - a short survey

Δ^4-Reductases

The properties of Δ^4-reductases, catalysing the irreversible reduction of the double bond between carbon 4 and 5, have been reviewed by Clark (1979). Because the reduction leads to the formation of an asymmetric carbon atom at C-5, either of two possible isomers, the 5α- or 5β-dihydro derivatives, may be formed. The Δ^4-5α-reductases are localized in liver microsomes whereas the Δ^4-5β-reductases are localized mainly in the soluble fraction of liver. The enzymes require NADPH as co-factor. The activity of Δ^4-reductases in extrahepatic tissues is very low, although detectable (Siebe et al., 1984; Hsia, 1971; Hartiala et al., 1979). Reduction of the 4,5-double bond is thought to be the rate limiting step in the metabolism of hydrocortisone and results in depletion of biological activity (Todd and Hechter, 1955; Glenn et al., 1957; Bush, 1962). Synthetic GCS, containing an additional double bond in the 1,2-position are, to a lesser extent, reduced by Δ^4-reductases (McGuire et al., 1960).

Hydrogenases - dehydrogenases

These enzymes catalyse the oxido-reduction of the hydroxyl/ketone pairs in the 3, 11 and 20 positions. Some properties were reviewed by Bush (1962). Apparently, these enzymes occur in many different forms with different substrate specificities. One such group of enzymes, with properties that distinguish them as a separate class, is the ketone reductases (Felsted and Bachur, 1980). $NADP^+$ is the preferred hydrogen acceptor. The hydrogenase - dehydrogenase activity is localized subcellulary in both the microsomal and soluble fractions, preferentially in the liver but to some extent also in other

tissues (Bush et al., 1967; Ward and Kasmarik, 1979; Siebe et al., 1984; Meigs and Engel, 1961). Ketone reductase activity is present even in erythrocytes (Cohen and Flockhart, 1975), a fact that probably explains why some steroid 20-keto, 21-carboxylic acid esters are biotransformed by 20-hydrogenation in human whole blood (unpublished observations). Reduction in the liver of the 3-keto group proceeds very rapidly after reduction of the 4,5-double bond has occurred. The 3-hydroxy group is then conjugated with glucuronic acid, thus drawing the reaction in the reductive direction (Bush, 1962). The interconversion of the 11β-hydroxy/keto group in hydrocortisone/cortisone and prednisolone/prednisone is well documented (Bush et al., 1968; Abramovitz et al., 1982; Nicholas and Lugg, 1982). Cortisone and prednisone have very low glucocorticoid activity and have to be converted by hydrogenases to hydrocortisone and prednisolone for therapeutic activity. Reduction of the 20-keto group is thought to result in depletion of activity (Bush, 1962), although exeptions have been reported (Lee and Soliman, 1982).

Monooxygenases (cytochrome P-450)

Cytochrome P-450 is an important hemoprotein participating in the oxidative metabolism of a wide variety of compounds including GCS (Estabrook et al., 1975; Kupfer and Orrenius, 1970). Hydroxylations catalysed by this enzyme involve the uptake of two electrons from NADPH with the reduction of one atom of O_2 to water and insertion of the other into the substrate. During the last 10 years it has become clear that cytochrome P-450 is not a single enzyme but a group of enzymes, each with different properties and substrate specificity (Lu and West, 1980). Furthermore, cytochrome P-450 is only the terminal enzyme participating in hydroxylation reactions. Other enzymes involved are NADPH-cytochrome P-450 reductase and cytochrome b_5 (Masters, 1980; White and Coon, 1980). In the literature, enzymes with these properties are often named monooxygenases or mixed function oxidases. However, it should be noted that all monooxygenase activity is not mediated by cytochrome P-450 (Ziegler, 1980).

The adrenal cortex is the richest source of cytochrome P-450 forms catalysing steroid hydroxylations, but the system is mainly concerned with the synthesis of endogenous steroid hormones. The monooxygenase system involved in the metabolism of foreign compounds, like synthetic GCS, is found primarily in liver microsomes, although some activity is also found in microsomes of lung, kidney, spleen, intestine and even skin (Litters et al., 1975; Masters, 1980). The monooxygenases catalyse 6β-hydroxylation and acetal side chain hydroxylation, the former being a very important inactivation reaction for most synthetic GCS. Thus, the 6β-hydroxy derivatives of hydrocortisone, triamcinolone acetonide, flunisolide and budesonide have glucocorticoid activities of less than 1% of the parent compounds (Kupfer, 1968; Kupfer et al., 1969; Chaplin et al., 1980_a; Dahlberg et al., 1984). Acetal side chain hydroxylation of budesonide and subsequent loss of the acetal moiety reduces the glucocorticoid activity to less than 0.3% of that of the parent drug (Dahlberg et al., 1984). Furthermore, the affinity of a stable acetal-hydroxylated derivative of budesonide (24-hydroxybudesonide) for the glucocorticoid receptor is less than 3% of that of budesonide (Axelsson, B., unpublished results).

Esterases

Esterases catalyse the hydrolytic cleavage of ester bonds. The properties of the non-specific carboxylesterases, which are thought to be involved in the hydrolysis of steroid esters, have been reviewed by Junge and Krisch (1975). These enzymes seem to be present in most biological tissues. Particulary high activities have been noted in liver and intestines (Hattori et al., 1981). According to Myers et al. (1982), choline esterases also appear to be able to catalyze the hydrolysis of steroid esters to some extent. Hydrolysis of the 17α-esters of beta-methasone and beclomethasone represents a deactivation step. The steroid 17α-alkohols have biological activities less than 0.5% of those of the corresponding esterfied compounds (Harris,

1975) as measured in the human vasoconstriction assay (McKenzie and Stoughton, 1962). However, the hydrolysis of beclomethasone 17α,21-dipropionate to beclomethasone 17α-propionate does not represent a real decrease in activity (Harris, 1975).

Conjugating enzymes

Glucuronyltransferase and sulfotransferase catalyse the conjugation of free hydroxy and carboxy groups of various substrates with glucuronic acid and sulfate, respectively. Particulary high activities are found in the liver. Glucuronyltransferase (review Kasper and Henton, 1980) is localized in microsomes and exists in at least two forms. In general, the enzyme has low affinity but high capacity for potential substrates. Sulfotransferase (review Jakoby et al., 1980) is a soluble enzyme that probably exists in many forms. Contrary to glucuronyltransferase, sulfotransferase has high affinity but low capacity towards acceptor substrates. Conjugation of GCS occurs mainly in the C-3 and C-21 positions; conjugation at C-3 requires the previous reduction of the 3-keto to the 3-hydroxy moiety. As concerns hydrocortisone, glucuronidation occurs in both the C-3 and C-21-positions and sulfatation in the C-21 position (Kornel et al., 1980). GCS conjugates are devoid of glucocorticoid activity.

Other enzymes

There is a group of enzymes that catalyses the oxidation of GCS to 21-carboxylic acids or 17β-carboxylic acids (review Monder and Bradlow, 1977). At present, the characteristics of these enzymes are less well known. Possibly, alcohol dehydrogenases (Bosron and Kai-Li, 1980) and aldehyde oxidizing enzymes (Weiner, 1980) may be involved. Carboxylic acids of GCS are generally devoid of glucocorticoid activity (Monder and Bradlow, 1980; Manz et al., 1983; Gorsline et al., 1985). However, esterfication yields surprisingly potent topical antiinflammatory agents having no detectable systemic activity (Gorsline et al., 1985; Manz et al., 1983).

Dehydrogenation of the B-ring has been reported for some GCS (Martinelli et al., 1979; Gordon and Morrison, 1978; Teitelbaum et al., 1981; Edsbäcker et al., 1986). As suggested by Teitelbaum et al. (1981), the mechanism involved may be linked to 6β-hydroxylation.

Studies on the relationship between metabolism and systemic activity of GCS

Kupfer et al. (1968; 1969; 1970), studied the induction of the liver enzyme tyrosine transaminase by some GCS. The pretreatment of rats with phenobarbital or 2,2-(o-chlorophenyl, p-chlorophenyl)-1,1-dichloroethane (o,p'DDD) markedly reduced the magnitude of induction by triamcinolone and triamcinolone acetonide. No such reduction of tyrosine transaminase induction by hydrocortisone was observed (Kupfer, 1968). On the other hand, pretreatment with SKF 525A substantially increased tyrosine transaminase induction by either triamcinolone and its acetonide (Kupfer, 1968). Since phenobarbital and o,p'DDD induce, and SKF 525A inhibits, the liver monooxygenase enzyme system responsible for the major metabolic reaction (6β-hydroxylation) of triamcinolone and its acetonide, it was concluded that these drug metabolizing enzymes are of physiological significance as modifiers of steroid action (Kupfer et al., 1969; Kupfer and Partridge, 1970). Contrary to triamcinolone and triamcinolone acetonide, hydrocortisone is biotransformed mainly by ring A-reducing enzymes, which are not induced by phenobarbital or o,p'DDD (Kupfer, 1968; Seutter, 1975).

It was shown in paper I that budesonide was about 4 times more rapidly biotransformed in liver preparations from male than from female rats. As observed in vivo, this was associated with a more pronounced thymus involuting activity of budesonide in female compared with male rats, especially after oral, but also to some extent after subcutaneous administration. Since budesonide has similar topical activity in both sexes, as measured in the rat ear edema test, it was suggested that drug

metabolizing enzymes are capable of reducing the systemic activity of budesonide. This was further supported in investigations by Brattsand et al.(1982_b), who found a 5-fold potentiation of budesonide in reducing thymus weight upon pretreatment of male rats with SKF 525A. The rapid biotransformation of budesonide observed in male rat livers is associated with a rapid 6β-hydroxylation. In female rat livers, budesonide is biotransformed mainly by ring A-reduction and 23-hydroxylation (Edsbäcker et al., 1986).

Influence of structural alterations on the metabolism of GCS

The 1,2-double bond

It has been shown that prednisolone is more slowly biotransformed than hydrocortisone by various rat liver preparations (Florini and Buyske, 1959; Schriefers et al., 1957). Further experiments indicated that the 1,2-double bond in prednisolone exerted this effect by slowing down ring A-reduction and possibly also reduction of the 20-keto group (Brown and Anason, 1958; Glenn et al., 1957). McGuire et al. (1960) reported that the 1,2-double bond completely prevented reduction by the microsomal Δ^4-5α-reductase of rat liver. Recovery of 5 times more unaltered prednisolone from human urine as could be recovered following administration of hydrocortisone supported increased metabolic stability of prednisolone in vivo (Sarett et al., 1963). As observed in dogs (Kuipers et al., 1957) and humans (Ely et al., 1956), prednisolone has a longer plasma half life than hydrocortisone. However, since similar major metabolites (ring A-reduced) of hydrocortisone and prednisolone could be identified in human urine after oral administration, the 1,2-double bond appeared not to induce a shift in metabolic pathways (Caspi and Pechet, 1958). Both hydrocortisone and prednisolone are only to a limited extent biotransformed by 6β-hydroxylation (Seutter, 1975).

In our investigations, we observed that 1,2-hydrogenation of prednisolone and budesonide enhanced the rate of biotransformation about 8-fold in rat liver but only 2-fold in human liver (Table 4). Moreover, the results indicated that the metabolites derived from hydrocortisone and 1,2-dihydrobudesonide in rat liver exhibited a fully reduced 3-keto,4-ene structure, whereas this was not the case with the metabolites of prednisolone and budesonide. This suggests that the 1,2-double bond reduces the rate of ring A reduction, and that the activities of the reducing enzymes are higher in rat liver than in human liver. In accordance with this, budesonide, having a $\Delta^{1,4}$,3-ene structure, is metabolized by ring A-reduction to a greater extent in rat liver than in human liver (Edsbäcker et al., 1986). The extremely rapid biotransformation noted for 1,2-dihydrobudesonide in rat liver may at least partly explain the low systemic activity observed with this substance when given to rats (Thalén et al., 1984).

The 16α-hydroxy group

The insertion of the 16α-hydroxy group in 9α-fluoroprednisolone was an important step in the early development of GCS (Freyberg et al., 1958). The compound triamcinolone proved to be highly effective in the control of rheumatoid arthritis. Florini and Buyske (1959) found that the 16α-hydroxy group reduced the rate of biotransformation approximately 2-fold in rat liver preparations which is in accordance with our results (Table 4). More important however, was the fact that the 16α-hydroxy group caused the formation of oxidised metabolites found in urine rather than reduced ones reported for hydrocortisone, prednisolone (Caspi and Pechet, 1958) and 9α-fluorohydrocortisone (Bush and Mahesh, 1958). Thus, in dog as well as in human urine about 20% of the given dose of triamcinolone could be accounted for as 6β-hydroxytriamcinolone and about 25% as unchanged triamcinolone (Florini et al., 1961_a and 1961_b). Only minor amounts of other unidentified metabolites were present in the urine. Thus, it appears as if the combination of

the 1,2-double bond, the 9α-fluoro atom and the 16α-hydroxy group efficiently inhibit reductive pathways leaving only the 6β-position exposed for oxidative attack. Considering our results (Table 4), it may be assumed that 6β-hydroxylation of triamcinolone as well as of fluocinolone and prednacinolone (16α-hydroxyprednisolone) occurs at a slow rate. It can be speculated that the reason for this is to be found in the highly polar nature of these GCS, which, as far as binding to monooxygenase enzymes is concerned, may be of disadvantage (Estabrook et al., 1975).

The 16α,17α-acetal group

The influence of acetal substitution on the rates of metabolism of GCS has now been studied for the first time. Substitution with the non-symmetric 16α,17α-acetal proved to be an efficient way to enhance the biotransformation rate in both rat and human liver (Table 4). In Fig.13 and 14, comparative metabolic disappearance curves for the symmetric and non-symmetric 16α,17α-acetal compounds are shown in comparison with the corresponding 16α,17α-hydroxy compounds. The major cause for the rapid biotransformation of the non-symmetric acetal GCS is the metabolic acetal side chain splitting of the 22R-epimers which was first shown with budesonide by Edsbäcker et al. (1983), and later reported for 9α-fluorobudesonide, 6α,9α-difluorobudesonide and 1,2-dihydrobudesonide (III). In the case of budesonide, we have been able to demonstrate that this reaction proceeds via a steroid ester intermediate after hydroxylation in the C-22 position of the acetal moiety. The ester intermediate is instantaneously hydrolysed to 16α-hydroxyprednisolone and butyric acid (Fig.10). This is a unique metabolic pathway not occuring with the symmetric acetal GCS nor with the 22S-epimers among the non-symmetric acetal GCS (Kripalani et al., 1975; Teitelbaum et al., 1981; Chu et al., 1979; Edsbäcker et al., 1983; III). Recently, however, it has been established that the 22S-epimer of budesonide is subjected to hydroxylation in the C-23 position of the acetal moiety

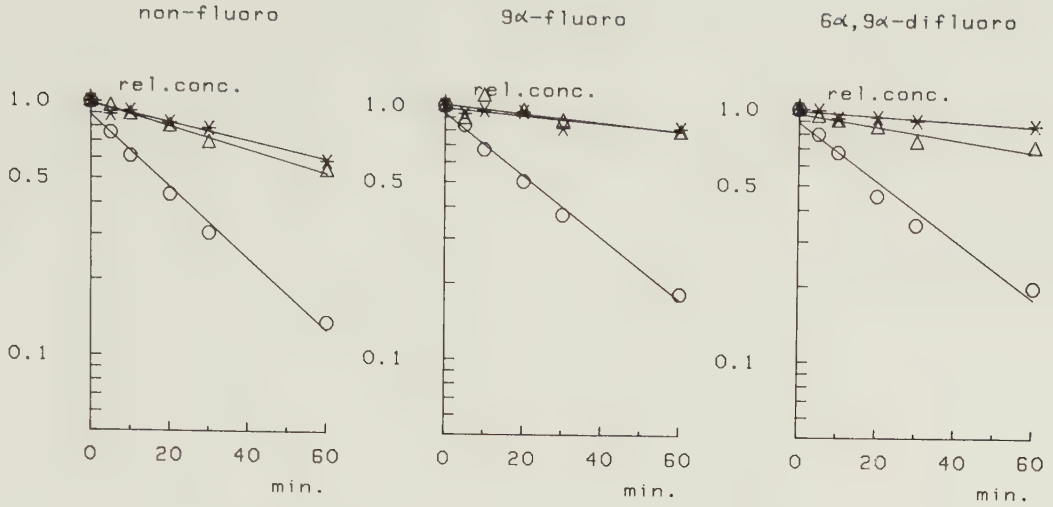


Fig.13. The metabolic disappearance of structurally related GCS during incubation with rat liver 9000 g supernatant fraction. Initial steroid conc.: 5 umol/l.

✱: 16α,17α-hydroxy GCS

△: symmetric 16α,17α-acetal GCS

○: non-symmetric 16α,17α-acetal GCS (22R + 22S)

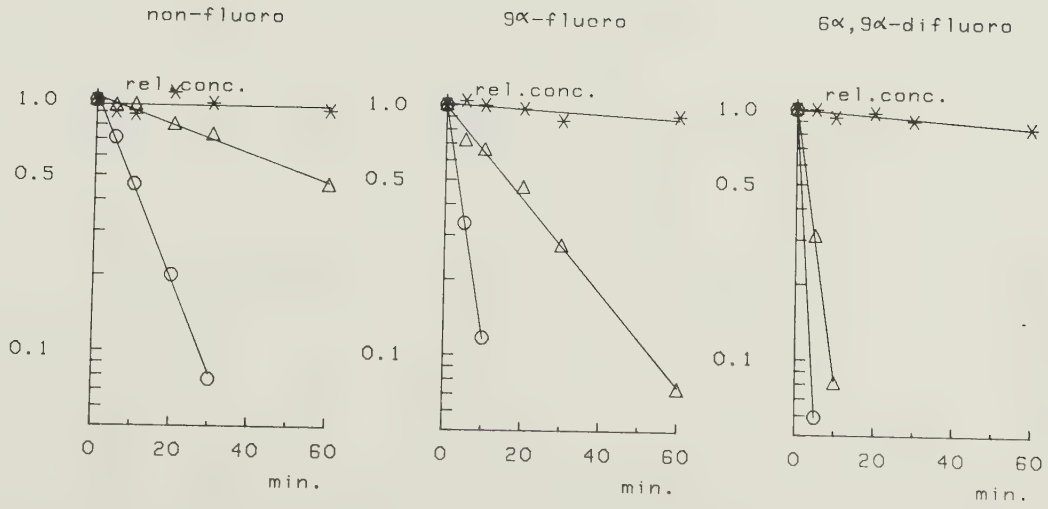


Fig.14. The metabolic disappearance of structurally related GCS during incubation with human liver 9000 g supernatant fraction. Initial steroid conc.: 5 umol/l.

✳: 16α,17α-hydroxy GCS

△: symmetric 16α,17α-acetal GCS

○: non-symmetric 16α,17α-acetal GCS (22R + 22S)

(Edsbäcker et al., 1986). This metabolic route does not result in loss of the acetal moiety. Both epimers of budesonide are furthermore rapidly biotransformed by 6β-hydroxylation in both rat and human liver.

A striking species difference was found with the symmetric acetal GCS. In rat liver, these drugs were slowly biotransformed, whereas this was not the case in human liver (Fig.13 and 14, Table 4). The major metabolic pathway of these GCS in both rat and man is 6β-hydroxylation (Teitelbaum et al., 1981; Kripalani et al., 1975; Gordon and Morrison, 1978; Chu et al., 1979; Chaplin et al.,1980$_a$).

The 6α- and 9α-fluoro atoms

The discovery of increased biological activity with steroid nucleus fluorination (Fried, 1957) initiated numerous investigations during the late fifties and early sixties. At that time it was generally believed that the fluoro atom caused this effect by slowing down the rates of inactivation of the substituted compounds. Indeed, Todd and Hechter (1955) found that 9α-fluorohydrocortisone exhibited a slightly decreased rate of biotransformation compared with hydrocortisone when incubated with rat liver preparations. Similar results were obtained by Glenn et al. (1957), Brown and Anason (1958) and Florini and Buyske (1959). Experiments with rat liver microsomes showed that this decrease in part was due to a retardation of ring A-reduction (McGuire et al., 1960). Another feature of the 9α-fluoro atom was discovered by Bush and Mahesh (1958), who demonstrated that the 9α-fluoro atom almost entirely prevented the formation of 11-keto metabolites upon oral administration of 9α-fluorohydrocortisone to man. This was later verified in vitro using rat liver microsomes (Bush and Hunter, 1961). Since the presence of an 11β-hydroxy group is considered essential for biological activity, this stabilizing effect of 9α-fluorination may be one cause for the elevated biological activity observed with the fluorinated GCS available

at the time. The extremely high topical activity of the GCS available today is thought to depend primarily on a high affinity for the glucocorticoid receptor (Dahlberg et al., 1984). Regardless of whether these topical GCS contain a 9α-fluoro atom or not, it has not been possible to demonstrate metabolic conversion of the 11-hydroxy group (Teitelbaum et al., 1981; Gordon and Morrison, 1978; Assandri et al., 1983; Edsbäcker et al, 1986). This is not entirely surprising, since these GCS contain a 1,2-double bond and C-16 substituents, a combination also known to prevent the formation of 11-keto metabolites (Bush et al., 1968).

Our results demonstrated that 6α,9α-fluorination of the 16α,17α-acetal GCS efficiently enhanced their rate of biotransformation in human liver (Fig.14 and Table 4), whereas fluorination had only marginal effect in rat liver (Fig.13 and Table 4). In rat liver preparations, the symmetric acetal GCS were biotransformed slowly, while in human liver the combination of fluorination and acetalization rendered these molecules highly susceptible to metabolism but not entirely in the order of the non-symmetric acetal GCS. To our knowledge this effect of fluorination has not been reported before for GCS.

It is tempting to assume that the enhanced rate of biotransformation at least of the 6α-fluorinated compounds in human liver, is explained by enhanced 6β-hydroxylation. Teitelbaum et al. (1981) reported that flunisolide (6α-fluorodesonide) was efficiently converted to 6β-hydroxydesonide by oxidative defluorination catalysed by mouse liver microsomal enzymes. The conversion proceeded via a stable 6-keto intermediate. The reaction was complicated by the formation of Δ^6-flunisolide, which was proposed as an alternative pathway upon oxidation in the 6β-position. We observed that the main metabolites derived from desonide and flunisolide had identical retention times on h.p.l.c., suggesting that 6β-hydroxydesonide was a common metabolite. Equivalent results were obtained when

the major metabolites derived from triamcinolone acetonide (9α-fluorodesonide) and fluocinolone acetonide (6α,9α-difluorodesonide) were compared. The identity of these metabolites was confirmed later upon comparison with authentic 6β-hydroxy-triamcinolone acetonide (6β-hydroxy, 9α-fluorodesonide). With this in mind, it could be shown that the 6α-fluoro atom to some extent enhanced the formation of the presumed 6β-hydroxy metabolite in human liver (Fig. 15). Opposite results were obtained in rat liver (Fig. 15).

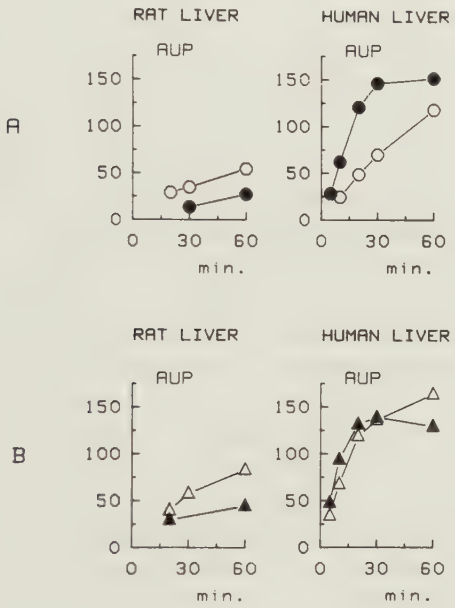


Fig.15. Relative rate of formation of 6β-hydroxydesonide (Fig.A) from desonide (○) or 6α-fluorodesonide (●) and of 6β-hydroxy, 9α-fluorodesonide (Fig.B) from 9α-fluorodesonide (△) or 6α,9α-difluorodesonide (▲) during incubation with rat and human liver 9000 g supernatant fraction (unpublished results).
AUP: area under uv-peak obtained by h.p.l.c.

Although the insertion of a 6α-fluoro atom may to some extent promote conversion to the 6β-hydroxy derivative, this is far from being the whole explanation for the rapid overall biotransformation of the fluorinated GCS observed in human liver. The role of the 9α-fluoro atom is also unclear. To fully explore the enhancing effect of fluorination on the rate of biotransformation, detailed studies with authentic 6β-hydroxy, 6-keto and Δ^6-compounds might be useful.

The 17α- and 21-ester groups

It is well known that steroids possessing a 21-ester group is more readily hydrolysed than those with a 17α-ester group (Nakano et al., 1981; O'Neill and Carless, 1980). The rate of ester cleavage varies with chain length. In the case of C-21 esters it has been reported that the optimum carbon number for maximum hydrolysis rate in the skin is around 4-5 (O'Neill and Carless, 1980). Hydrolysis of 17α-esters seems to be sterically hindered by substituents present in the C-16 position (Nakano et al., 1981). Before being hydrolysed these compounds must undergo non-enzymatic rearrangment via a cyclic orthoester intermediate to the corresponding 21-ester compounds (Misaki et al, 1982). This rearrangment has been studied in detail in aqueous solution by Bundgaard and Hansen (1981) and Anderson et al. (1984). It is also well known that species differences regarding hydrolysis of steroid esters exist, the rat being a far more rapid hydrolyser than man (Weisenberger, 1972; Misaki et al., 1982).

During incubation of beclomethasone 17α,21-dipropionate with human liver and lung preparations, the compound was rapidly hydrolysed to beclomethasone 17α-propionate (IV), a compound possessing biological activity comparable with the dipropionate derivative (Harris, 1975). The monoester was then further hydrolysed in the lung to beclomethasone, but at a much slower rate. Upon incubation with human liver preparation, no beclomethasone could be detected, and in comparison with

budesonide, beclomethasone 17α-propionate was 2-4 times less rapidly biotransformed (Table 5). Of various 17α-esters, valerate esters were 9-10 times more rapidly biotransformed than the corresponding propionate esters by human liver preparation (Table 6). Furthermore, fluorination in the 9α-position enhanced their rate of biotransformation approximately 2-fold. Only small amounts of the expected hydrolysis products were detected. Instead another metabolite with unknown structure was formed. This indicates that 17α-esters of GCS, at least those having a 16β-methyl group, are biotransformed in the liver mainly by oxidative and possibly also reductive pathways.

In vitro - in vivo considerations

The majority of the work presented in this thesis has been carried out in vitro with liver 9000 g supernatant fractions. Presumably, this fraction contains most of the important liver enzymes functional in the metabolism of foreign compounds. In accordance with this, the metabolite pattern obtained after incubation of budesonide conforms qualitatively with that obtained in plasma after intravenous administration of the drug to man (Edsbäcker et al., 1983).

Incubation of GCS with liver preparations might be useful in attempts to predict their systemic bioavailability after oral administration. Bioavailability (F) can be calculated as F=1-E, where E is the fraction of the drug administered that is lost before entering the general circulation. Provided quantitative absorption from the gastro-intestinal tract and negligible metabolism in the intestinal mucosa, which seems to be the case with most GCS, E is determined by the first pass metabolism in the liver. In Table 8, available data on bioavailability of some relevant GCS are given.

Table 8. Systemic bioavailability (F) of some GCS after oral administration.

Compound	Species	F(%)	Reference
Prednisolone	man	78-85	Bergrem et al. (1983)
	man	>80	Täuber et al. (1984)
1,2-Dihydro-	man	40-71	Toothaker et al. (1982)
prednisolone	rat	~100	Chanoine, Junien (1984)
6α-Fluorodesonide	man	21	Chaplin et al. (1980_a)
	rat	~100	Chu et al. (1979)
Budesonide (22R+22S)	man	11	Ryrfeldt et al. (1982)
	rat	10-30	Andersson, unpublished
Budesonide (22R)	man	8	Borgå, unpublished
Budesonide (22S)	man	18	Borgå, unpublished

With the exeption of the figure (100%) given for 1,2-dihydro-prednisolone (hydrocortisone) in rats, these figures are in accordance with our in vitro data (Table 4) in the sense that a rapid liver biotransformation observed in vitro correlates with a high hepatic first pass metabolism and consequently a low bioavailability. The reason for the divergence concerning the hydrocortisone metabolism in rat liver is not known.

Metabolic in vitro studies may be of help in order to choose species for pharmacological and toxicological evaluation of potential drugs. Brattsand et al. (1982_a) demonstrated that the introduction of a non-symmetric 16α,17α-acetal group in GCS markedly enhanced the topical antiinflammatory activity compared with that of the conventional symmetric acetal group. This is thought to depend on a higher receptor affinity of the non-symmetric acetal GCS (Dahlberg et al., 1984). However, despite this both types of acetal compounds had a similar

systemic glucocorticoid activity in rat models (Brattsand et al., 1982_a). The major cause of this might be the high metabolic stability of the symmetric acetal GCS observed on incubation with rat liver (Fig.13). In human liver, on the other hand, the symmetric acetal compounds are readily biotransformed (Fig.14), suggesting a more favourable therapeutic ratio in humans than in rats. This has also been verified clinically with aerosolized triamcinolone acetonide and flunisolide (Webb, 1976; Chaplin et al., 1980_b). Apparently, data obtained with acetal GCS using rat models should be interpreted with care, especially when extrapolations of animal data to man are to be considered. From a metabolic point of view, the mouse seems to be a more relevant species for pharmacological and toxicological evaluations of acetal GCS (II; Takai et al., 1982; Edsbäcker et al., 1986).

Inter-individual differences in man

While the rank order of biotransformation rate of the studied GCS was the same in all individual human livers, marked differences of the metabolic capacities existed between the livers - some livers were slow others fast. These differences were pronounced in the case of the synthetic steroids budesonide, triamcinolone acetonide and beclomethasone 17α-propionate but not with hydrocortisone (Fig.16). The highest rate of biotransformation of the synthetic GCS was obtained upon incubations with supernatant fractions of livers 9 and 13. Genetic differences between the donor patients may be one explanation. However, it is interesting to note that livers 9 and 13 were obtained from subjects who had received repeated doses of the anticonvulsant agent phenytoin during the last week before death (Table 9). Phenytoin is a potent inducer of drug metabolising enzymes involved in the oxidative metabolism of eg. GCS (Jubiz and Meikle, 1979; Park and Breckenridge, 1981). As mentioned earlier, budesonide and triamcinolone acetonide are biotransformed predominantly by monooxygenase catalysed reactions whereas the primary metabolic pathways of

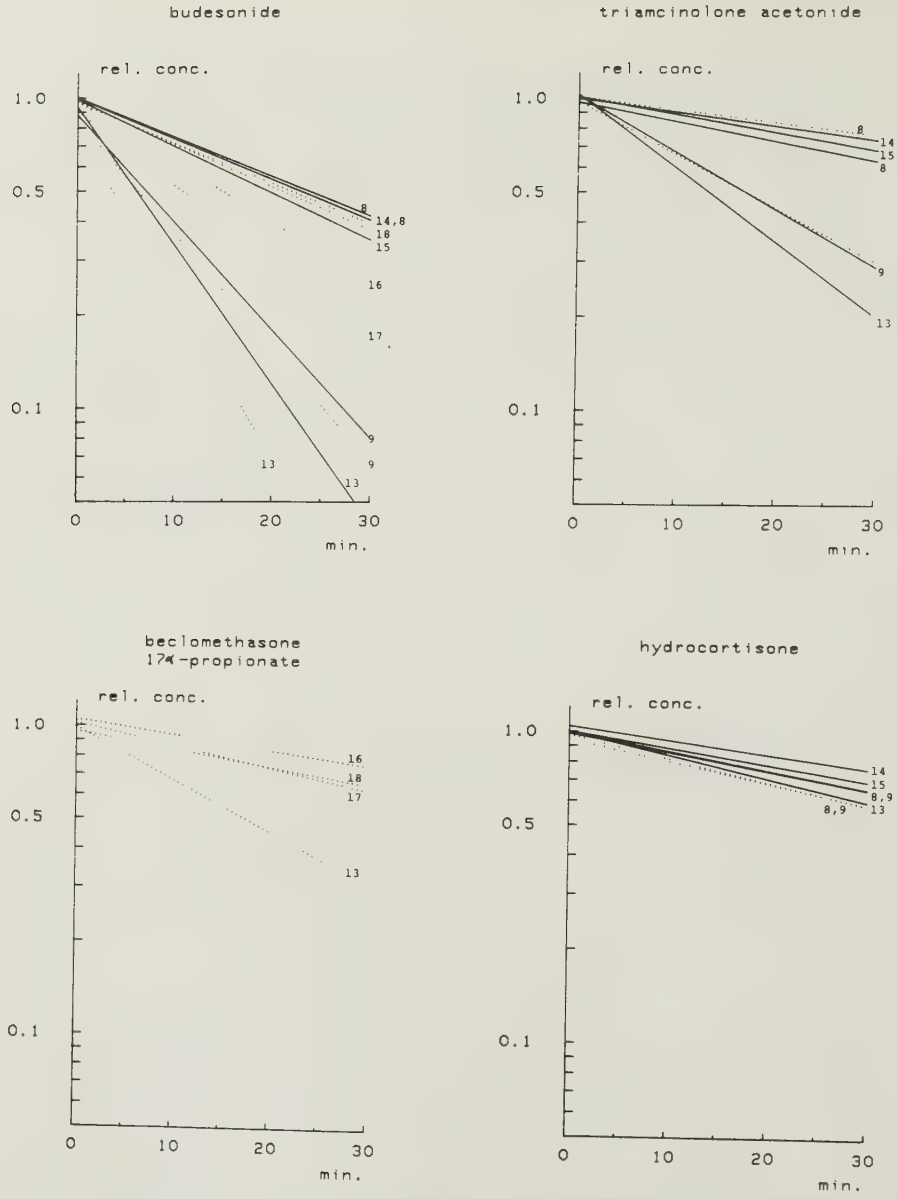


Fig.16. The metabolic disappearance of some tritium labelled GCS during incubation with individual human liver 9000 g supernatant fractions. The livers are numbered according to von Bahr.
Incubation concentration: 1.0 umol/l (continuous lines)
0.1 umol/l (dotted lines)

Table 9. Data on liver donor patients and liver microsomal cytochrome P-450 concentration (from von Bahr et al., 1982 and personal communication).

Sample	Sex,age	Known relevant drug intake last week	Cyt.P-450 nmol/mg prot.
8	M,49	single doses of lidocaine and dexamethasone	0.45
9	M,43	repeated doses of phenytoin and pentazocine. A few doses of paracetamol, diazepam, pancuronium and phenoperidine.	0.61
13	F,53	repeated doses of phenytoin, diazepam and pentazocine. Few doses of aminocaproic acid, dexamethasone and paracetamol.	0.73
14	M,18	no drug intake	0.39
15	F,40	few doses of dexamethasone.	0.26
16	M,52	few doses of betamethasone	0.47
17	M,24	--	0.46
18	F,59	few doses of dexamethasone	0.70

hydrocortisone are reductive and not induced by phenytoin (Jubiz and Meikle, 1979; Seutter, 1975). These observations may have important clinical implications. No correlation seemed to exist between the rate of biotransformation of the GCS and the total concentration of liver cytochrome P-450 (Table 9). This is not entirely surprising since the liver is known to contain a mixure of different forms of cytochrome P-450 (Lu and West, 1980), that may differ between individuals.

Tissue distribution of budesonide

The topical GCS budesonide has successfully been introduced for treatment of asthma, rhinitis and skin diseases. The high therapeutic and low systemic activity observed with this drug when given by aerosol has allowed substantial reduction or withdrawal of oral steroid therapy (Rosenhall et al., 1982). There is no doubt that the efficient liver biotransformation of budesonide contributes to these favourable properties. Recently, however, some interest has been focused on tissue distribution as another important factor for the relationship between therapeutic effects and side effects of GCS (Edsbäcker, 1986).

Budesonide is rapidly absorbed from the lung, although a small fraction is retained in the lung for a longer period of time (Ryrfeldt et al., 1982; 1986). Budesonide is not thought to be biotransformed to significant extent in the lung (IV; Ryrfeldt et al., 1986). About 10% of the fraction that is deposited in the stomach during inhalation therapy will escape the first pass metabolism and thus enters the general circulation (Ryrfeldt et al., 1982). The absorbed fraction, whether it comes from the lung or the gastro-intestinal tract will induce systemic side effects unless distributed in a favourable way.

The tissue distribution and fate of budesonide in vivo was studied in mouse, which metabolically is closer to man than rat is. After intravenous administration, tritiated budesonide rapidly escaped the blood and was concentrated in various tissues

(Fig.11 and 12). Five min after dosing, the liver contained as much as 23% of the radioactive dose given. Only 30% of this fraction could be attributed to unchanged budesonide, while the remaining 70% was polar metabolites reflecting the efficient liver biotransformation of budesonide. Considering the tissue/blood concentration ratio of unchanged budesonide, the kidneys, the spleen and particularly the lungs showed high values (ratios of 22-53) after distribution equilibrium had been attained (after 40 min, Table 7). A distribution of budesonide to liver and lung is highly favourable as these organs represent the site of loss (liver) and the site of action (lung). The brain/blood concentration ratio of budesonide never exceeded 5, suggesting an operating blood-brain barrier (Table 7). As demonstrated by whole body autoradiography, the brain radioactivity was mainly localized to the cerebellum (Fig.11) and not to functional areas involved in the regulation of adrenal function with the exception of a distinct but rapidly fading uptake of radioactivity in the pituitary. Low levels in blood and brain of administered GCS should ensure a minimal effect on the HPA-axis.

CONCLUDING REMARKS

In order to predict the usefulness of a potential drug in humans, the therapeutic activity and toxicity have to be evaluated in relevant animal models. To select species for these models, it has become increasingly vital that metabolic and kinetic data are available during an early stage of drug development. In vitro studies may here play an important role. During the last years, the availability of fresh human tissue for drug metabolism studies has been a valuable complement.

The development of topical GCS has so far been focused on getting as high therapeutic activity as possible and at the same time lowering the adverse systemic effects. In this respect, the

topical GCS budesonide is a good example. The unique non-symmetric 16α,17α-acetal substituent present in this drug increases the topical activity and provides at the same time sites for metabolic inactivation in the liver. As has been shown, 6α- and 9α-fluorination will also enhance the metabolic rate of GCS in humans. Substituents that will reduce the rate of metabolism includes the 1,2-double bond and the 16α-hydroxy group. To be fully aware of the consequences of structural alterations, one should always consider the relative biological activity and toxicity of the metabolites formed as well as the overall kinetics and distribution of the potential drug. It is hoped that the results presented in this thesis may be of use in the future development of even more selective GCS.

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BIOTRANSFORMATION RATE (*IN VITRO*) AND SYSTEMIC POTENCY (*IN VIVO*) OF THE TOPICAL GLUCOCORTICOID BUDESONIDE IN MALE AND FEMALE RATS

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SUMMARY

Budesonide is a glucocorticoid of clinical interest for the topical treatment of skin and respiratory diseases. The *in vitro* liver biotransformation rate and *in vivo* systemic potency (thymus involution) of budesonide were studied in male and female rats. The biotransformation rate of [^{3}H]-budesonide was about 4 times slower in the female than in the male rat liver 9000 *g* supernatant ($t_{1/2}$: 230 and 57 min, respectively). The systemic potency of budesonide after peroral or subcutaneous administration was higher (by factors of 6 and 2, respectively) in the female than in the male rat. These results suggest that the liver biotransformation rate of budesonide is of great importance in reducing its systemic action in the male rat.

INTRODUCTION

Budesonide (Fig. 1) is a new potent non-halogenated glucocorticoid suitable for topical treatment of respiratory (aerosol treatment) and skin diseases. In animal models it has been observed that budesonide has a favourable ratio between topical and systemic activities [1, 2]. The high topical potency is probably due to a new type of 16,17-acetal substitution [2]. The rapid biotransformation rate of budesonide in the liver compared with that of triamcinolone acetonide and hydrocortisone as shown in *in vitro* experiments may explain its relatively low systemic activity [3]. Earlier experiments in rats by Kupfer *et al.*[4–6] have shown that the systemic activity of triamcinolone acetonide is related to the monooxygenase activity of the liver. It was therefore of interest to investigate if this also is true for budesonide. Sex differences in the biotransformation of several drugs including glucocorticoids have been demonstrated in rats [7–13]. Briefly, male rats have a higher capacity to biotransform drugs than female rats, *in vivo* as well as *in vitro*. Thus the rat may serve as a model for investigations concerning the relationship between biotransformation and systemic activity of drugs.

The present study demonstrates a pronounced sex difference in the biotransformation rate of budesonide in the 9000 *g* rat liver supernatant. The rate was closely correlated to the systemic glucocorticoid activity in the two sexes.

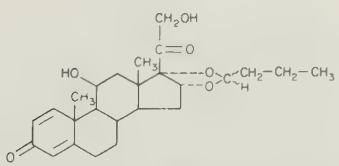

Fig. 1. Structural formula of budesonide.

MATERIALS AND METHODS

Materials

[1,2-^{3}H]-budesonide (16α,17α-Butylidenedioxy-11β, 21-dihydroxy-[1,2-$^3H_2$]-pregna-1,4-diene-3,20-dione), TRQ 1343, SA: 78 mCi/mg 2.89 GBq/mg) was obtained from the Radiochemical Centre, Amersham, England. Prior to the incubation experiment, [^{3}H]-budesonide was purified by preparative chromatography on a Lipidex 5000® gel (Packard) with heptane–chloroform (7:3, v/v) as mobile phase. Subsequent analytical chromatography (HPLC, description below) revealed that the radiochemical purity of the compound was greater than 95%. Non-labelled budesonide was supplied by Dr A. Thalén, AB Draco. Water was Millipore-filtered (Millipore Super-Q 4 module system). Chloroform was distilled. Dichloromethane, ethanol, heptane were of spectroscopic grade. All solvents (obtained from Kebo Grave, Malmö, Sweden) were degassed before use. D-Glucose-6-phosphate, NADP and glucose-6-phosphate dehydrogenase were obtained from Sigma. Male and female Sprague–Dawley rats, weighing about 200 *g*, were obtained from K. E. Möllegård, Ejby, Denmark.

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Biotransformation rate (in vitro) *in the 9000 g liver supernatant*

The animals (3 × 5 female rats and 3 × 5 male rats) were decapitated and the livers quickly perfused *in situ* with the same buffer as used in the homogenization procedure (below). In groups of five from the same sex, the rat livers were removed and transferred into cups kept on ice. The livers were homogenized in 2.5 vol. of a K_2HPO_4–KH_2PO_4 buffer (0.1 M, pH 7.4, also containing 0.2 M sucrose) with a Teflon pestle (10 strokes) operating at 3000 rev/min. The homogenate was then centrifuged for 20 min at 9000 *g*. The resulting supernatant was used in the incubation. The incubations were performed at 37°C in the presence of carbogen gas (95% O_2 + 5% CO_2). Each incubation medium (vol.: 6 ml) also contained 6 mg of protein, 60 μmol $MgCl_2$, 45 μmol of glucose-6-phosphate, 1.8 μmol of NADP and 2.5 units of glucose-6-phosphate dehydrogenase. After an equilibrium period of 10 min at 37°C, the incubations were started by the addition of [^{3}H]-budesonide (dissolved in 60 μl ethanol) to produce the initial incubation concentration of 10^{-6} mol/l. Aliquots (0.5 ml) were taken at 0, 15, 30, 45, 60 and 90 min of incubation. The samples were immediately frozen in dry ice (−60°C). An internal standard solution (1.5 ml containing 60 μg unlabelled budesonide) was added to each sample. Unchanged budesonide was then extracted with 4 ml of dichloromethane by gentle shaking for 30 min followed by centrifugation for 15 min at 2000 rev./min. The organic phase (3 ml) was collected and dried under a gentle stream of nitrogen. The residue was then dissolved in 200 μl of ethanol–water (45:55, v/v). By extraction of simulated incubation samples, a recovery of 97.4% ± 1.2% (mean ± SD, $n = 8$) of [^{3}H]-budesonide was obtained. Unchanged [^{3}H]-budesonide was analysed by high performance liquid chromatography (HPLC) as described earlier [3].

Radioactivity was measured by the liquid scintillation technique. The measurements were performed in Instagel® (Packard) using a PA 3255 (Packard) liquid scintillation counter, equipped with external standard for estimation of counting efficiency.

Systemic potency (in vivo)

The systemic potency of budesonide was studied after oral and subcutaneous administration to male and female rats. Budesonide was suspended in a vehicle (CMC-Na 0.75 g, Tween 80 0.04 g and NaCl 0.7% w/w ad 100 g) and homogenized in a Turrax blender. The suspension was injected subcutaneously on the back or fed by stomach tube in a volume of 0.1–0.5 ml/rat. Reduction of thymus weight was determined being one of the most sensitive parameters for systemic glucocorticoid activity. At least four doses (Tables 1 and 2) of budesonide were investigated with five rats per group for each sex and each route of administration.

Table 1. Sex difference in thymus involution potency of budesonide after p.o. administration to rats

Sex	Dose (mg/kg)	Thymus weight, mg (mean ± SEM)	ED_{50} (mg/kg)
Male	Vehicle	646 ± 21	
	0.22	636 ± 39	
	0.67	599 ± 37	2.54
	2.0	354 ± 59**	(1.97 3.40)
	6.0	159 ± 18***	
Female	Vehicle	480 ± 26	
	0.06	494 ± 17	
	0.11	383 ± 42	
	0.22	296 ± 49*	0.43
	0.44	239 ± 47**	(0.32 0.67)
	0.89	169 ± 25***	

*, ** resp. *** = $P < 0.05$, 0.01 resp 0.001 in comparison to vehicle group.
( ) = 95% confidence limits.

Table 2. Sex difference in thymus involution potency of budesonide after s.c. administration to rats

Sex	Dose (mg/kg)	Thymus weight, mg (mean ± SEM)	ED_{50} (mg/kg)
Male	Vehicle	700 ± 35	
	0.07	577 ± 35*	
	0.22	445 ± 38**	0.33
	0.67	196 ± 23***	(0.27–0.40)
	2.00	78 ± 5***	
Female	Vehicle	487 ± 28	
	0.05	385 ± 45	
	0.11	325 ± 19**	0.19
	0.22	241 ± 36***	(0.14–0.27)
	0.44	124 ± 34***	

*, **, resp *** = $P < 0.05$, 0.1 resp 0.001 in comparison to vehicle group.
( ) = 95% confidence limits.

The ED_{50} was defined as the dose, which reduced the thymus weight to 50% of that in the vehicle group. On the linear part of the dose-response curve, the ED_{50} was calculated by linear regression analysis, as described elsewhere [14].

RESULTS AND DISCUSSION

Aerosolized glucocorticoids are used e.g., in the topical treatment of lung diseases such as asthma. The amount of drug deposited in the lung is rapidly absorbed into the blood [15, 16]. However, in aerosol therapy a substantial fraction of the drug is also deposited in the oral cavity, swallowed and then absorbed from the gastro-intestinal tract [15]. The absorbed drug fraction can induce systemic side effects [17]. Systemic effects with glucocorticoids have also been observed during topical treatment of skin disorders [18]. One way to minimize these undesired effects is to develop glucocorticoids which are extensively inactivated in the liver by biotransformation.

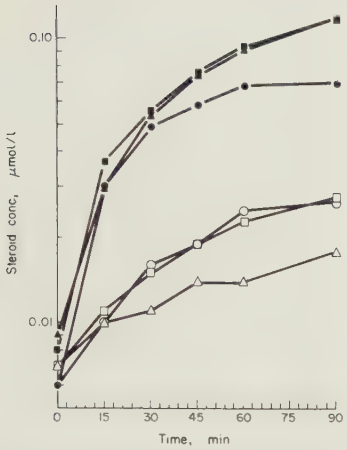

Fig. 2. The formation of non-extractable radioactivity (expressed as unchanged drug) during incubation of tritiated budesonide with male (filled symbols) and female (open symbols) rat liver *in vitro*. ○: experiment 1. △: experiment 2. □: experiment 3.

Budesonide is intended for topical treatment of skin and respiratory diseases. In animal models, low systemic activity has been observed [2], very probably as a consequence of its inactivation by a rapid liver biotransformation [3]. The key role of the liver is also supported by *in vitro* studies with rat lung and kidney where no biotransformation of budesonide could be detected [16, and Andersson P. *et al.*, unpublished investigation]. More detailed studies about the relationship between liver biotransformation and systemic activity of budesonide are, however, necessary. The present study was undertaken with rats in which, unlike other species, differences between the sexes in the biotransformation rate of drugs are known to occur.

Tritiated budesonide was incubated with the 9000 *g* liver supernatant from both male and female rats. The radioactivity remaining in the aqueous phase after extraction with dichloromethane was measured in each sample taken from the incubation medium. This non-extractable radioactivity represents polar metabolites derived from [^{3}H]-budesonide. This fraction increased with incubation time, more rapidly in the medium from male than from female livers (Fig. 2), indicating a more extensive biotransformation of [^{3}H]-budesonide in the male rat liver. HPLC analyses of unchanged [^{3}H]-budesonide confirmed this finding. Thus the disappearance of [^{3}H]-budesonide was more rapid in male rat liver (Fig. 3). Since the degradation seemed to follow first order kinetics, the half life ($t_{1/2}$) of [^{3}H]-budesonide in the different incubation media was used to characterize the metabolic rate. The mean $t_{1/2}$ was 57 min (range 48–72, $n = 3$) in male liver, and 230 min (range 192–213, $n = 3$) in female liver. This sex difference is similar to that found with other glucocorticoids [11–13], and is probably caused by a higher activity of oxidative enzymes present in male livers [11].

In the *in vivo* studies, budesonide was administered in various doses both perorally and subcutaneously to female and male rats and reduction of the thymus weight was determined as a parameter for systemic potency. The ED_{50}-values are given in Tables 1 and 2. After peroral administration (Table 1) budesonide was about 6 times more potent in female rats ($ED_{50} = 0.43$ mg/kg) than in male rats ($ED_{50} = 2.54$ mg/kg). After subcutaneous administration (Table 2), budesonide was about twice as potent in female ($ED_{50} = 0.19$ mg/kg) as in male rats ($ED_{50} = 0.33$ mg/kg). The sex difference in thymolytic activity might theoretically be explained by different activity at the receptor level. This is, however, not supported by the investigation of the topical anti-inflammatory activity where both sexes have a similar sensitivity for budesonide (Brattsand *et al.*, unpublished investigations). Instead it is suggested that the more rapid biotransformation of budesonide in male rat liver explains its greater systemic tolerance in that sex. The sex difference in systemic activity activity was most pronounced after peroral administration. However, in both sexes, the absolute systemic activity of budesonide was highest after subcutaneous administration. These results are most readily explained by an extensive first-pass metabolism of budesonide in the liver after peroral administration. If this explanation is valid, the thymus involution data indicate a six

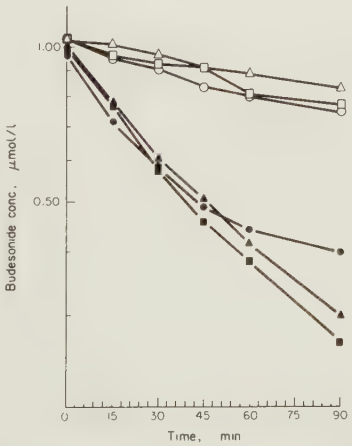

Fig. 3. The degradation of tritiated budesonide by male (filled symbols) and female (open symbols) rat liver *in vitro*. ○: experiment 1. △: experiment 2. □: experiment 3.

times higher oral bioavailability in the female compared to the male rat.

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IN VITRO BIOTRANSFORMATION OF GLUCOCORTICOIDS IN LIVER AND SKIN HOMOGENATE FRACTION FROM MAN, RAT AND HAIRLESS MOUSE

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SUMMARY

The pharmacological effects of glucocorticoids are greatly influenced by their pharmacokinetic properties. In the present report, the *in vitro* biotransformation of the topical glucocorticoids [^{3}H]-budesonide ([^{3}H]-BUD), [^{3}H]-triamcinolone acetonide ([^{3}H]-TAAc) and [^{3}H]-hydrocortisone ([^{3}H]-HC) was studied in the 9000 *g* liver and skin supernatant from man, rat and hairless mouse. The rate of disappearance of the compounds was estimated during the initial 30 min of incubation by high performance liquid chromatography. In human liver the half life ($t_{1/2}$) rank order was [^{3}H]-BUD (7–23 min) < [^{3}H]-TAAc (13–68 min) < [^{3}H]-HC (40–67 min), in rat liver [^{3}H]-HC (14–21 min) < [^{3}H]-BUD (28–38 min) < [^{3}H]-TAAc (161–196 min) and in hairless mouse liver [^{3}H]-BUD (17–22 min) < [^{3}H]-TAAc (21–34 min) < [^{3}H]-HC (82–165 min). Negligible biotransformation of these glucocorticoids occurred in skin.

BUD is a one to one mixture of the [22R]- and [22S]-epimers. It was found that the [22R]-epimer was more susceptible to liver biotransformation than the [22S]-epimer of [^{3}H]-BUD.

The results are discussed with particular reference to the extent of systemic side effects of these compounds.

INTRODUCTION

Ever since hydrocortisone was discovered to be efficacious against diseases exhibiting an inflammatory reaction, the synthesis of more potent glucocorticoids has continued. Such glucocorticoids are especially of interest in the local treatment of respiratory and skin diseases, since high potency is required for a rapid and efficient therapeutic response. However, the increase in potency has increased the risks of systemic glucocorticoid effects. This may be due to a high intrinsic activity and a slow systemic inactivation of such compounds [1]. Glucocorticoids used in local therapy should therefore be rapidly inactivated after absorption into the systemic circulation to avoid the development of systemic side effects.

Budesonide (Fig. 1, BUD) is a new potent, non-halogenated glucocorticoid that has a favourable ratio between local and systemic effects [2]. The pharmacokinetics of BUD as described in dogs [3] may provide an explanation for this. BUD is a one to one mixture of two epimers with [22R] and [22S] configuration [4], which both seem to have about the same qualitative pharmacological effects. Epimer [22R] has, however, a somewhat higher topical anti-inflammatory potency than epimer [22S] [5].

The aim of the present investigation was to compare the biotransformation rate of BUD *in vitro* with that of hydrocortisone (Fig. 1, HC) and triamcinolone acetonide (Fig. 1, TAAc) in 9000 *g* liver supernatant from man, rat and hairless mouse [6]. Hydrocortisone is an endogenous, non-halogenated steroid with weak potency while TAAc is a halogenated, highly potent, synthetic steroid. The biotransformation rates of the two epimers of BUD were also compared. The liver is considered to be the most important organ for glucocorticoid biotransformation. The 9000 *g* liver supernatant fraction is suitable for this kind of study because it contains both microsomal and soluble enzymes responsible for the biotransformation of glucocorticoids. Since the skin is one important target organ in the topical treatment with glucocorticoids, it was also of interest to investigate whether enzymatic degradation of the compounds occurs in 9000 *g* skin supernatant.

MATERIALS AND METHODS

Chemicals

Tritiated budesonide ([1,2-^{3}H]-BUD (11β,21-dihydroxy-16α,17α-[(22R,S)-propylmethylenedioxy]-[1,2-$^3H_2$]-pregna-1,4-diene-3,20-dione) TRQ

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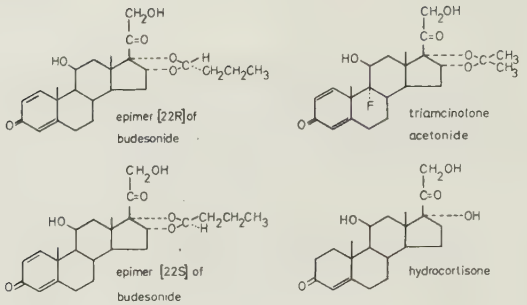

Fig. 1. Structural formulas of budesonide (a 1:1 mixture of epimers [22R] and [22S]), triamcinolone acetonide and hydrocortisone.

1341, specific activity: 97 mCi/mg, 3.59 GBq/mg), triamcinolone acetonide ([1,2,4-^{3}H]-TAAc) (9α-fluoro - 11β,21 - dihydroxy - 16α,17α - [(1 - methylethylidene)dioxy] - [1,2,4 - $^3H_3$] - pregna - 1,4 - diene - 3,20-dione), TRK 1293, specific activity: 53 mCi/mg, 1.96 GBq/mg) and hydrocortisone ([1,2^{3}H]-HC (11β,17α,21 - trihydroxy - 4 - [1,2 - $^3H_2$] - pregnene-3,20-dione), TRK 133, specific activity 114 mCi/mg, 4.22 GBq/mg) were obtained from the Radiochemical Centre, Amersham, England. Prior to the incubation experiments, [^{3}H]-BUD and [^{3}H]-TAAc were purified by preparative chromatography on a Lipidex 5000® gel (Packard) with heptane–chloroform (70:30, v/v) as mobile phase. [^{3}H]-HC was purified by chromatography on a Sephadex LH-20® gel (Pharmacia) with cyclohexane–ethanol (80:20, v/v) as mobile phase. Subsequent analytical chromatography (HPLC, description below), revealed that the radiochemical purity of the compounds was greater than 95%. The epimeric ratio ([22R]:[22S]) for [^{3}H]-BUD was 48:52.

Non-labelled BUD and TAAc were supplied by Dr A. Thalén, AB Draco, and non-labelled HC was from Apoteksbolaget, Sweden.

Water was Millipore-filtered (Millipore Super-Q 4 module system). Chloroform was redistilled. Dichloromethane, heptane, ethanol and cyclohexane were of spectroscopic grade. All solvents (obtained from Kebo-Grave, Malmö, Sweden) were degassed before use. D-Glucose-6-phosphate, NADP and glucose-6-phosphate dehydrogenase were obtained from Sigma, Stockholm, Sweden.

Preparation of 9000 g liver and skin supernatant

Male Sprague–Dawley rats (weight: 200 g) were obtained from K. E. Möllegård, Ejby, Denmark. Male hairless mice (hr/hr-Bom, weight: 25 g) were obtained from G. L. Bomholtgård, Ry, Denmark. The mice were killed by cervical dislocation and the rats by decapitation. The livers were perfused *in situ* with the same buffer as used in the homogenization procedure (below). The liver and skin (dorsal) were quickly removed and kept at +4°C until homogenised one hour later. One normal human skin sample was taken from the breast during a mastectomy at Lund hospital, Sweden and stored at −80°C before use. Five human liver samples (livers 1–5) were supplied from the liver bank at Huddinge Hospital, Sweden (7; supported by the Swedish Medical Research Council no. 14X-05667). Livers 1, 2 and 4 were taken from male (age: 49, 43 and 18), livers 3 and 5 from female (age: 53 and 40) cadaveric renal transplant donors shortly after circulatory arrest and immediately frozen at −196°C. They were then stored at −80°C before use. Storage under these conditions preserves most enzyme activities for at least 6 months [7].

All subcutaneous tissues were removed from the skin. The skin specimens were then minced and homogenized for 15 s in 10 vol. of a K_2HPO_4–KH_2PO_4 buffer (0.1 M, pH 7.4, also containing 0.2 M sucrose) with a Polytron PT 20 homogenizer (Kinematica, Lucerne, Switzerland). The connective tissue was removed by filtration through gauze and then the homogenate was centrifuged for 20 min at 9000 ***g***. The resulting supernatant was used in the incubation experiment.

The livers from the rats and hairless mice were homogenized in 2.5 vol. of homogenizing buffer with a teflon pestle (10 strokes) operating at 3000 rev./min. The homogenate was then centrifuged for 20 min at 9000 ***g***. The resulting supernatant was used in the incubation experiment.

The human livers were homogenized for 15 s in 2.5 vol. of homogenizing buffer with a Polytron PT 20 homogenizer. The homogenate was filtered through 2–3 layers of gauze to remove connective tissue. The filtrate was further homogenized by 10 strokes with a Teflon pestle operating at 3000 rev./min, and then centrifuged at 9000 ***g***. The resulting supernatant was used in the incubation experiment.

The protein concentration of the supernatants was estimated according to Lowry *et al.*[8]. Bovine serum albumin was used as standard. The supernatants were diluted to 1 mg protein/ml.

Incubation

Each glucocorticoid was incubated at concentrations of 10^{-7} and 10^{-6} mol/l with the 9000 *g* liver supernatants and at a concentration of 10^{-7} mol/l with the 9000 *g* skin supernatants. Each 9000 *g* incubation medium from rat and hairless mouse was prepared from 5 animals, while each 9000 *g* incubation medium from man was prepared from one single individual. The incubations were performed at 37°C in the presence of carbogen gas (95% O_2 + 5% CO_2). Each incubation medium (vol.: 6 ml) also contained 6 mg of protein, 60 μmol of $MgCl_2$, 45 μmol of glucose-6-phosphate, 1.8 μmol of NADP and 2.5 units of glucose-6-phosphate dehydrogenase. In order to check non-enzymatic degradation during the experiment, blank incubations with heated (70°C) supernatant were also made. All incubations were performed in borosilicate flasks (25 ml). After an equilibrium period of 10 min at 37°C the incubations were started by the addition of the tritiated glucocorticoids.

Sampling and extraction

Aliquots (0.5 ml) were taken from each incubation flask and transferred into borosilicate test tubes after 0, 6, 15, 30, 45, 60 and 90 min of incubation. The samples were immediately frozen in dry ice (−60°C). At the same time, aliquots (100 μl) were taken for measurements of total radioactivity.

The extraction of unchanged glucocorticoid and the addition of an internal standard were performed according to Ryrfeldt *et al.*[3]. To each sample, 1.5 ml of an internal standard solution and 4 ml of dichloromethane were added. In the BUD analysis, an internal standard solution containing 40 μg of unlabelled BUD/ml was used. In the TAAc and HC analyses, the corresponding internal standard solutions contained 20 μg of unlabelled TAAc/ml and 25 μg of unlabelled HC/ml, respectively. The extraction was made by gentle shaking for 30 min followed by centrifugation for 15 min at 2000 rev./min. The aqueous phase was analyzed for total radioactivity. The organic phase (3.0 ml) was collected and dried under a gentle stream of nitrogen. The residue was then dissolved in 200 μl of the mobile phase used in HPLC. By extraction of simulated incubation samples, recoveries of 97.4% (SD 1.2%, $n = 8$, [^{3}H]-BUD), 99.1% (SD 2.1%, $n = 7$, [^{3}H]-TAAc) and 86.2% (SD 3.0%, $n = 8$, [^{3}H]-HC) were obtained.

High performance liquid chromatography

The liquid chromatograph consisted of a Waters M 6000 A pump, a Valco injector equipped with a 30 μl loop, and a Waters M 440 u.v. detector (254 nm) connected to a Tarkan W + W 600 recorder. The outlet of the detector was interconnected with a LKB Redirac fraction collector.

The epimers of BUD separate in a reversed phase system which has been described by Wikby *et al.*[9]. In the present study, the analyses of BUD and its epimers were performed on Nucleosil C_{18} bonded phase (5 μm), on 5 × 200 mm column with ethanol–H_2O (45:55, v/v) as mobile phase. The flow rate was 1.0 ml/min. This modified system gave an almost complete separation of the epimers of BUD, which allowed quantification of each of them. The analyses of TAAc were performed with ethanol–H_2O (40:60, v/v) as mobile phase. The analyses of HC were made on a Nuceosil NO_2 bonded phase (5 μm), on a 5 × 200 mm column [10], with dichloromethane–ethanol (47:3, v/v) as mobile phase at a flow rate of 3 ml/min. All columns were slurry packed. The effluent was collected in scintillation vials and assayed for radioactivity. To assure an optimal separation of the epimers of [^{3}H]-BUD and at the same time to minimize the number of scintillation vials, different fraction volumes were collected (Fig. 2C). The UV-peak of the internal standard was recorded and the ratio of radioactivity of the tritium-labelled drug peak to the area of the internal standard u.v.-peak was calculated. The u.v.-areas of the labelled compounds are negligible compared to the u.v.-areas of the internal standards. Before each analysis, the analytical system was standardized. Three concentrations of the tritiated corticosteroids (in the range $0.1–1.0 \times 10^{-6}$ mol/l or $0.1–1.0 \times 10^{-7}$ mol/l) in heated (70°C) 9000 *g* skin or liver supernatant were chosen. A standard curve was produced. All standard curves had a correlation coefficient >0.999.

Measurements of radioactivity

Radioactivity was measured by the liquid scintillation technique. The measurements were performed in Instagel® (Packard) using a PA 3255 (Packard) liquid scintillation counter. The counting efficiency was estimated by means of the external standard channel ratio procedure.

RESULTS

In each sample taken from the incubation medium, the radioactivity present both in the resulting water and organic phases after extraction with dichloromethane was measured. During incubation with liver from all species, the non-extractable radioactivity increased with incubation time. Simultaneously, the extractable radioactivity decreased. This indicates the formation of polar metabolites from all three glucocorticoids. The non-extractable radioactivity formed during 30 min of incubation with human liver is given in Table 1. During incubation with skin from all species, the ratio between non-extractable and extractable radioactivity remained unchanged with time (0.02 (BUD), 0.01 (TAAc) and 0.10 (HC)), indicating negligible formation of polar metabolites of the glucocorticoids in skin.

The analyses of unchanged glucocorticoids in the incubation samples were performed by HPLC as described in Materials and Methods. Figures 2A–C show the HPLC elution profiles of radioactivity after

Table 1. Non-extractable radioactivity (expressed as % of total radioactivity) formed during 30 min of incubation of tritiated glucocorticoids with human liver (*in vitro*)

	Non-extractable radioactivity (%)						
	Liver 1		Liver 2		Liver 3	Liver 4	Liver 5
	10^{-7}	10^{-6}	10^{-7}	10^{-6}	10^{-6}	10^{-6}	10^{-6}
HC	7.4	6.4	10.3	7.6	9.4	6.5	5.8
TAAc	6.2	6.2	17.1	19.3	23.0	5.5	7.8
BUD	15.6	16.6	28.9	28.7	31.2	17.1	21.2

Initial glucocorticoid concentrations: 10^{-7} and 10^{-6} mol/l.
HC: hydrocortisone, TAAc: triamcinolone acetonide, BUD: budesonide.

90 min of incubation of the glucocorticoids with human liver No. 1. Similar radioactive patterns were obtained with all human livers. The figures represent the radioactivity extractable into dichloromethane. In the reversed phase chromatography system used in the TAAc and BUD analyses, the metabolites were eluted before the unchanged drug, and in the straight phase chromatography system used in the HC analysis, the metabolites were eluted after the unchanged drug. The metabolites of all three drugs are therefore considered to be more polar than the parent compounds. The metabolites might have been formed through oxidative, reductive and conjugative pathways.

The disappearance of [^{3}H]-glucocorticoid with time was studied in human liver (Fig. 3A–E), rat liver (Fig. 4A) and hairless mouse liver (Fig. 4B) incubation medium. The initial glucocorticoid concentration was 10^{-6} mol/l. Similar curves were obtained at an initial glucocorticoid concentration of 10^{-7} mol/ml. The biotransformation rates of all glucocorticoids seemed to follow first order kinetics during the first 30 min. During the period from 30–90 min, a deviation from linearity was noted. This may be due to formed metabolites, which compete with unchanged drug at active sites of the liver enzymes and/or, in the case of hairless mouse, interfere in the analyses of unchanged BUD (see below). For comparative purposes, the half

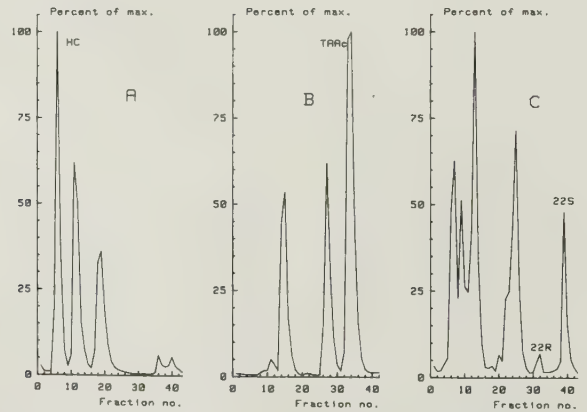

Fig. 2. Elution profiles of radioactivity after chromatography of a dichloromethane extracted incubation sample of [^{3}H]-hydrocortisone (HC, Fig. A), [^{3}H]-triamcinolone acetonide (TAAc, Fig. B) and [^{3}H]-budesonide (Fig. C) with human liver 1 (incubation time: 90 min) at an initial glucocorticoid concentration of 10^{-7} mol/l. Fig. A. Column: Nucleosil NO_2 (5 μm), mobile phase: dichloromethane–ethanol (94:6, v/v), flow: 3.0 ml/min, fraction volume: 0.3 ml (fraction No. 1–35), 3.0 ml (fraction No. 36–43). Fig. B Column: Nucleosil C-18 (5 μm), mobile phase: ethanol–H_2O (40:60, v/v), flow: 1.0 ml/min, fraction volume: 0.4 ml (fraction No. 1–43). Fig. C. Column: Nucleosil C-18 (5 μm), mobile phase: ethanol/H_2O (45/55), flow: 1.0 ml/min, fraction volume: 0.5 ml (fraction No. 1–30), 0.8 ml (fraction No. 31–32), 0.2 ml (fraction No. 33–38) and 1.0 ml (fraction No. 39–43). 22R = epimer [22R] of BUD. 22S = epimer [22S] of BUD.

life ($t_{1/2}$) during the first 30 min was calculated (Tables 2 and 3). No biotransformation of the glucocorticoids occurred in the heated (70°C) 9000 *g* liver supernatants.

In human liver (Fig. 3A–E and Table 2) the order of biotransformation rate was [^{3}H]-BUD > [^{3}H]-TAAc > [^{3}H]-HC and in rat liver (Fig. 4A and Table 3) [^{3}H]-HC > [^{3}H]-BUD > [^{3}H]-TAAc. In the analyses of [^{3}H]-BUD in liver from hairless mouse, metabolite interference in the chromatography peaks for both the epimers [22R] and [22S] was found. The concentration of [^{3}H]-BUD in the liver incubate from this species therefore represents [^{3}H]-BUD plus these metabolites. However, the order of biotransformation rate was [^{3}H]-BUD (including the interfering metabolites) > [^{3}H]-TAAc > [^{3}H]-HC (Fig. 4B and Table 3). In liver from all species, the [22R]-epimer was more susceptible to biotransformation than the [22S]-epimer of [^{3}H]-BUD. This epimer difference was most pronounced in hairless mouse liver (Tables 2 and 3).

The incubation experiments with skin from all species showed that little or no biotransformation of the [^{3}H]-glucocorticoids occurred. Figures 5A–C show

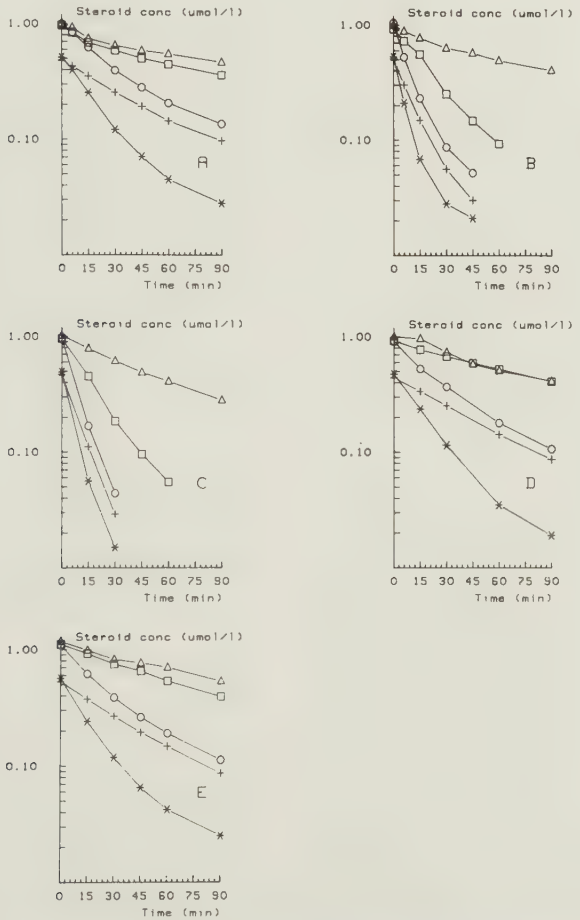

Fig. 3. Concentration of unchanged tritiated hydrocortisone (△), triamcinolone acetonide (□), budesonide (○), epimer [22R] (*) and epimer [22S] (+) of budesonide during incubation with human liver 1–5 (Figs A–E) *in vitro*. Initial glucocorticoid concentration: 10^{-6} mol/l.

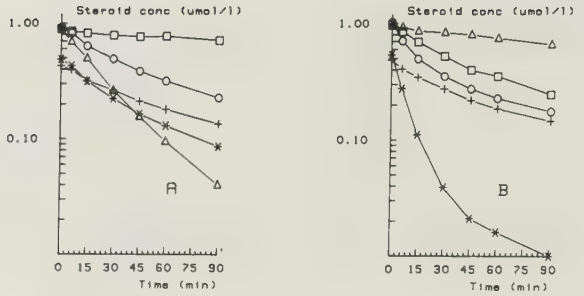

Fig. 4. Concentration of unchanged tritiated hydrocortisone (△), triamcinolone acetonide (□), budesonide (○), epimer [22R] [*] and epimer [22S] (+) of budesonide during incubation with rat (Fig. A) and hairless mouse liver (figure B) *in vitro*. Initial glucocorticoid concentration: 10^{-6} mol/l. Mean of two experiments.

the concentration time curve of unchanged glucocorticoid during incubation with human, rat and hairless mouse skin. In addition, no metabolites from the glucocorticoids could be detected.

DISCUSSION

In the treatment of various dermatoses, the skin is the target organ for drug therapy. The topical potency of a drug is theoretically determined by its intrinsic activity, penetration rate, biotransformation rate and retention in the skin. The relative importance of these factors has not yet been fully evaluated. The drug fraction that is absorbed into the systemic circulation may induce systemic side effects. In the case of glucocorticoids with high topical potency, it has been observed that these side effects can be pronounced [11]. The development of BUD has shown that it is poss-

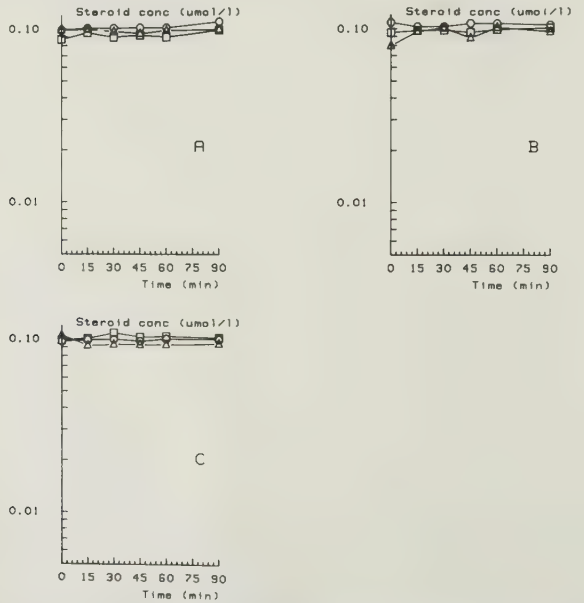

Fig. 5. Concentration of unchanged tritiated hydrocortisone (△), triamcinolone acetonide (□) and budesonide (○) during incubation with human (Fig. A), rat (Fig. B) and hairless mouse skin (Fig. C) *in vitro*. Initial glucocorticoid concentration: 10^{-7} mol/l.

Table 2. The *in vitro* half life (min) of tritiated glucocorticoids during a 30 min incubation with human liver

	Glucocorticoid half life (min)						
	Liver 1		Liver 2		Liver 3	Liver 4	Liver 5
	10^{-7}	10^{-6}	10^{-7}	10^{-6}	10^{-6}	10^{-6}	10^{-6}
HC	40	46	42	47	41	67	58
TAAc	68	43	17	16	13	67	53
BUD	22	22	8	9	7	23	20
Ep [22R]	16	14	6	7	6	15	13
Ep [22S]	31	32	9	10	8	38	30

Initial glucocorticoid concentrations 10^{-7} and 10^{-6} mol/l.
HC: hydrocortisone, TAAc: triamcinolone acetonide, BUD: budesonide, Ep [22R]: epimer [22R] of budesonide, Ep [22S]: epimer [22S] of budesonide.

ible to design glucocorticoids with high topical potency but with relatively less potency to induce systemic glucocorticoid action [2]. This differentiation may be due to a high intrinsic glucocorticoid activity of BUD in the skin and its rapid systemic biotransformation, leading to metabolites of much lower intrinsic activity. The present study was made to compare BUD's biotransformation rate *in vitro* with that of HC and TAAc in liver and skin from man, rat and hairless mouse.

In the present study, no measurable biotransformation of the [^{3}H]-glucocorticoids investigated was found in the skin of any species during 90 min of incubation (Figs 5A–C). This is partly in accordance with Hsia and co-authors [12, 13, 14], who reported that only 8% of HC was metabolised during an incubation period of 4 h with chopped human skin from different anatomical regions, and at an initial HC concentration comparable to that used in the present investigation. They also identified the resulting metabolites and found qualitative differences between the various skin regions [12]. However, Hsia *et al.* did not investigate human breast skin, which was used in the present investigation. If our results are also relevant for the *in vivo* situation, the glucocorticoid fraction that is systemically absorbed after epicutaneous application very probably has most of its biologic activity preserved. Therefore, to avoid systemic side effects, it is desirable that these compounds are rapidly inactivated in the liver. This is especially important for BUD and TAAc since these two glucocorticoids have a much higher intrinsic activity than HC.

Table 3. The *in vitro* half life (min) of tritiated glucocorticoids during a 30 min incubation with rat and hairless mouse liver

	Glucocorticoid half life (min)			
	Rat liver		Hairless mouse liver	
	10^{-7}	10^{-6}	10^{-7}	10^{-6}
HC exp 1	17	14	82	165
HC exp 2	—	21	113	140
TAAc exp 1	196	161	24	32
TAAc exp 2	—	186	21	34
BUD exp 1	33	28	22	20
BUD exp 2		38	17	20
Ep [22R] exp 1	25	23	8	8
Ep [22R] exp 2		29	7	8
Ep [22S] exp 1	44	36	36	37
Ep [22S] exp 2		48	26	38

Initial glucocorticoid concentrations: 10^{-7} and 10^{-6} mol/l.
HC: hydrocortisone, TAAc: triamcinolone acetonide, BUD: budesonide, Ep [22R]: epimer [22R] of budesonide, Ep [22S]: epimer [22S] of budesonide.

The results from the incubation experiments with liver show that the glucocorticoids investigated were rapidly metabolised by liver enzymes (Tables 2 and 3). This indicates the importance of the liver in reducing side effects. In all human livers studied, the order of biotransformation rate was [^{3}H]-BUD > [^{3}H]-TAAc > [^{3}H]-HC (Figs 3A–E). However, while [^{3}H]-HC was biotransformed at about the same rate in all human livers, the biotransformation of [^{3}H]-BUD and [^{3}H]-TAAc was about three times as fast in human livers 2 and 3 compared with the other livers (Table 2). One explanation for this may be genetic differences. However, induction of metabolism is also likely since livers 2 and 3 were obtained from humans, who one week before death received repeated doses of pentazocine and phenytoin [7], the latter being an enzyme inducer. In contrast to HC, which is mainly biotransformed by reductive pathways [1], most synthetic glucocorticoids, e.g. TAAc, are mainly biotransformed by oxidative pathways [15–18]. Phenytoin is known to induce the biotransformation of synthetic glucocorticoids to a greater extent than it does the biotransformation of HC [19].

It is of special interest to compare the ratio between local and systemic effects of TAAc and BUD, because both these compounds represent potent topical glucocorticoids of 16α,17α-acetal structure (Fig. 1). The

topical potency of BUD in human skin, as assayed by the vasoconstriction test, is about three times higher than that of TAAc [5]. Since in the present investigation it was found that [^{3}H]-BUD was more susceptible to human liver biotransformation than [^{3}H]-TAAc, it is suggested that BUD may have a more favourable ratio between local and systemic effects than TAAc in humans. However, it must be remembered that other factors than biotransformation, e.g. plasma protein binding, distribution volume and excretion rate also influence the biological effects of drugs *in vivo*.

BUD is a 1:1 mixture of two epimers ([22R] and [22S]) [4]. It was found that the rapid initial biotransformation rate of [^{3}H]-BUD in human liver was mainly due to the rapid biotransformation of the [22R]-epimer. While epimer [22S] was biotransformed at a rate about twice as fast as [^{3}H]-TAAc, the [22R]-epimer was biotransformed twice as fast as epimer [22S] (Table 2). Interestingly, in the human vasoconstriction test, epimer [22R] is about twice as potent as epimer [22S] of BUD [5]. The relative systemic activity of the two epimers in man is unknown at present.

In the experiments with rat liver, [^{3}H]-BUD was biotransformed more rapidly than [^{3}H]-TAAc (Fig. 4A). The topical to systemic activity of these two compounds in the rat has been investigated by Brattsand *et al.* The topical potency of BUD to inhibit rat ear edema is about three times that of TAAc [5]. However, with respect to systemic side effects as exemplified by the reduction of thymus weight, TAAc is about five times as potent as BUD after epicutaneous administration. Similar relationships between BUD and TAAc in reducing thymus weight were found after oral and subcutaneous administration [5]. As [^{3}H]-BUD was biotransformed five times more rapidly than [^{3}H]-TAAc (Table 3), it is suggested that a more rapid liver biotransformation of BUD is the explanation of its demonstrated improved relation between local and systemic effects. In the rat, the difference in liver biotransformation rate between the two epimers of [^{3}H]-BUD was not as pronounced as in human liver. Epimer [22R] was degraded 1.5 times as fast as epimer [22S] (Table 3). In the rat (*in vivo*), epimer [22R] is more potent than epimer [22S] with respect to both topical anti-inflammatory and to systemic glucocorticoid activity [5]. The biotransformation of [^{3}H]-HC in rat liver was more rapid than for the two other glucocorticoids (Fig. 4A). This is in contrast to the findings in the experiments with human liver.

In liver from hairless mouse, the order of degradation rate of the [^{3}H]-glucocorticoids was similar to that of human liver: [^{3}H]-BUD > [^{3}H]-TAAc > [^{3}H]-HC (Fig. 4B). However, in the quantification of [^{3}H]-BUD in liver from this species, there was interference from a metabolite which was more pronounced at longer incubation times. The actual biotransformation of [^{3}H]-BUD in liver from hairless mouse is therefore underestimated. In this liver there was a pronounced difference in biotransformation rate between the two epimers of [^{3}H]-BUD. Epimer [22R] was biotransformed about five times as fast as epimer [22S] (Table 3).

This study demonstrates species differences in the liver biotransformation rate of glucocorticoids *in vitro*. However, in all species studied, [^{3}H]-BUD was more susceptible to liver biotransformation than [^{3}H]-TAAc. The more rapid biotransformation of [^{3}H]-BUD may be due to its lack of a 9α-fluoro atom (Fig. 1), a substituent present in the [^{3}H]-TAAc molecule (Fig. 1). 9α-Fluoro-substitution is known to stabilize the 11-OH group of glucocorticoids against metabolic attack [20]. Additional sites in the steroid nucleus may also be stabilized by the halogen. It is also likely that the difference between the two steroid structures with respect to their substituents in the 16,17-acetal group influence their biotransformation rates (Fig. 1). The influence of the configuration at C-22 is shown by the observed difference in biotransformation rates between the two opimers of BUD.

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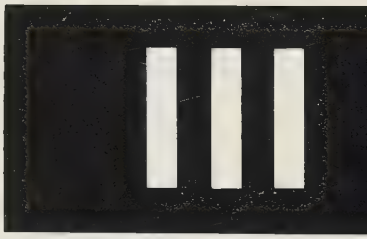

EFFECT OF STRUCTURAL ALTERATIONS ON THE BIOTRANSFORMATION RATE OF GLUCOCORTICOSTEROIDS IN RAT AND HUMAN LIVER

Running title: Liver metabolism of glucocorticoids

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Footnote

Part of these data have been presented at the Ninth European Workshop on Drug Metabolism, 1984, Nancy, France.

Key words: biotransformation, in vitro, liver, rat, human, glucocorticosteroids

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ABSTRACT

1. The effect of structural alterations on the biotransformation rate of glucocorticosteroids (GCS) in rat and human liver 9000 g supernatant fraction was studied.
2. Insertion of a 16α-hydroxy group in the prednisolone molecule (16α-hydroxyprednisolone) was found to decrease the rate of biotransformation. Substitution of the 16α,17α-hydroxy groups with a symmetric acetal (in e.g. desonide) or especially a non-symmetric acetal (in e.g. budesonide), enhanced the biotransformation rate several-fold, particularly in human liver.
3. Differences in the rates of metabolism in rat and human liver were observed. Hydrogenation of the 1,2-double bond in prednisolone and budesonide (hydrocortisone and 1,2-dihydrobudesonide) enhanced the biotransformation rate 9-fold in rat liver but only 2-fold in human liver. Fluorination of the steroid nucleus in 6α- and 9α-positions enhanced the biotransformation rate several-fold in human liver but in rat liver, fluorination marginally decreased the rate of biotransformation.
4. These in vitro results correlate well with available data on the first pass liver metabolism of the studied GCS. This indicates that in vitro data can be useful in predicting oral bioavailability of GCS.

INTRODUCTION

The systemic activity of glucocorticosteroids (GCS) is partly dependent on the rate and extent of their inactivation in the liver (e.g. Kupfer, 1968; Andersson et al. 1982a). A low first pass metabolism in liver can be of advantage for GCS intended for oral systemic treatment (e.g. prednisolone). However, in the local treatment of asthma with topical GCS (e.g. aerosolized budesonide, flunisolide, triamcinolone acetonide and beclomethasone dipropionate) it is essential that the systemic effects are minimized. In such therapy the major part of the discharged dose is swallowed and then absorbed from the gastrointestinal tract (Newman et al, 1981; Davies, 1982). Thus the systemic effects can be reduced by an efficient first pass metabolism in the liver. In the search for new topical GCS drugs it is therefore of importance to consider the influence of structure on the biotransformation of these drugs.

We now report the effect of different substituents on the overall biotransformation rate of GCS in rat and human liver 9000 g supernatant fraction. The basic structural formulas are given in figure 1. Starting with prednisolone, the effect of a 16α-hydroxy substituent (16α-hydroxyprednisolone), a symmetric 16α,17α-acetal substituent (desonide)

and a non-symmetric 16α,17α-acetal substituent (budesonide) were studied. Budesonide is a mixture of two epimers with 22R and 22S configuration (Albertsson et al. 1978). These epimers were investigated separately. In addition, the effect of hydrogenation (in 1,2-position) of prednisolone and budesonide and of fluorination (in 6α-, 9α-positions) of 16α-hydroxyprednisolone, desonide and budesonide was studied.

EXPERIMENTAL

Materials: The 22R- and 22S-epimers of budesonide 1,2-dihydrobudesonide, 9α-fluorobudesonide and 6α,9α-difluorobudesonide were prepared by resolution on Sephadex LH-20 of the corresponding epimeric mixtures (Thalén and Nylander, 1982). Desonide, 9α-fluorodesonide (triamcinolone acetonide), 6α-fluorodesonide (flunisolide), 6α,9α-difluorodesonide (fluocinolone acetonide), 16α-hydroxyprednisolone, 9α-fluoro, 16α-hydroxyprednisolone (triamcinolone) and 6α,9α-difluoro, 16α-hydroxyprednisolone (fluocinolone) were obtained from LARK S.p.A., Milan, Italy. Prednisolone and 1,2-dihydroprednisolone (hydrocortisone) were obtained from Sigma Company, Sweden. Tritiated budesonide (^{3}H in the 1,2-position, TRQ 2280), triamcinolone acetonide (^{3}H in the 1,2,4-position, TRQ 1293) and prednisolone (^{3}H in the 2,4,6,7-position, TRQ 691) were obtained from the Radiochemical Centre, Amersham, England. The specific activity was 94 (^{3}H-budesonide), 46 (^{3}H-triamcinolone acetonide) and 162 mCi/mg (^{3}H-prednisolone). Analytical chromatography (h.p.l.c.) showed a radiochemical purity of the compounds greater than 90%. Water was filtered with a Millipore membrane. Ethanol was of spectroscopic grade. D-Glucose 6-phosphate, NADPH, and glucose 6-phosphate dehydrogenase were obtained from Sigma Chemical Company, Sweden.

Preparation of the 9000 g liver supernatant fraction: The livers from seven male Sprague Dawley rats (body weight 207-228 g, K.E. Möllegård, Ejby, Denmark) were removed immediately after decapitation. The livers were pooled and homogenized in 2.5 volumes of K_2HPO_4-KH_2PO_4 buffer (0.1 M, pH: 7.4, also containing 0.2 M of sucrose) with a Teflon pestle (10 strokes) operating at 3000 rev/min. The homogenate was then centrifuged for 20 minutes at 9000 g.

Six human liver samples (samples 15, 16, 17, 18, 20 and 21) were obtained from the liver bank at Huddinge Hospital, Huddinge, Sweden (von Bahr et al., 1980). The samples were taken from renal transplant donors shortly after circulatory arrest and immediately frozen at -196°C. The sex and age of the donors were F40, M52, M24, F59, F56 and M65, respectively. The liver samples were pooled and homogenized for 15 sec in 2.5 volumes of buffer with a Polytron PT20 homogenizer and then with a teflon pestle (10 strokes) operating at 3000 rev/min. The homogenate was then centrifuged at 9000 g for 20 min. All preparations with both rat and human livers were carried out at +4°C or below. The protein concentration was estimated according to Lowry et al. (1951). The homogenates were stored at -80°C until incubation. Storage under these conditions during the experiment was found not to alter the enzymic activity.

Incubation: The incubations of the GCS were performed on three separate occasions. On the first occasion, budesonide, 9α-fluorobudesonide, 6α,9α-difluorobudesonide, desonide, 9α-fluorodesonide, 6α-fluorodesonide, 6α,9α-difluoro-desonide and hydrocortisone were incubated. On the second occasion, prednisolone, hydrocortisone, budesonide and 1,2-dihydrobudesonide were incubated and on the third occasion 16α-hydroxyprednisolone, triamcinolone and fluocinolone were incubated. All GCS were incubated with exactly the same incubation medium (9000 g supernatant fraction) on all occasions at an initial concentration of 5 μM. The incubations were performed at 37°C in the presence of carbogen gas (95% O_2 and 5% CO_2). Each incubation medium contained 42 mg of protein, 140 μmol of $MgCl_2$, 315 μmol of Glucose 6-phosphate, 12.6 μmol of NADP and 17.5 units of glucose 6-phosphate dehydrogenase in a total volume of 14 ml. In order to check non-enzymic degradation during the experiment, incubations with denaturated supernatants (heated at 65°C) were also made. All incubations were performed in borosilicate flasks (50 ml). After an equilibrium period for 10 min at 37°C the incubations were started by addition of the GCS dissolved in 140 μl of ethanol. Aliquots (2.0 ml) were taken from each incubation flask and transferred into borosilicate test tubes at 0, 5, 10, 20, 30 and 60 min of incubation. The samples were immediately frozen on dry ice (-60°C) and then kept at -20°C until extraction.

Extraction: The extraction procedure was performed at +4°C. After thawing, 8 ml of an internal standard solution (containing either 9 nmol of the Draco steroid D-5086 or 9.6 nmol of desonide or 10 nmol of hydrocortisone in 10% of ethanol and 0.01 M of acetic acid) was added to all samples. As a recovery indicator either ^{3}H-budesonide (12.4 pmol), ^{3}H-triamcinolone acetonide (10.0 pmol) or ^{3}H-prednisolone (3.4 pmol) was included in the internal standard solution. Nine ml of the mixture (total volume: 10 ml) was applied on a pre-conditioned Sep-Pak^{R}, C_{18} cartridge (Waters Associates). After washing the cartridge with 10 ml of 0.01 M of acetic acid, the steroids were eluted with 4 ml of ethanol - 0.01 M of acetic acid (60:40 v/v). The eluate was diluted with 0.01 M of acetic acid to a final ethanol concentration of 8% and then applied on a second pre-conditioned Sep-Pak, C_{18} cartridge. After washing the cartridge with 10 ml of ethanol - 0.01 M acetic acid (8:92 v/v) the steroids were eluted with 4 ml of ethanol. By measuring the radioactivity, recoveries of 94.8 $\pm$ 3.3% ($\bar{X} \pm$ S.D., n=70, rat liver), 94.1 $\pm$ 3.0% ($\bar{X} \pm$ S.D., n=72, human liver) of ^{3}H-budesonide, and 95.0 $\pm$ 2.2% ($\bar{X} \pm$ S.D., n=46, rat liver) and 95.2 $\pm$ 2.6% ($\bar{X} \pm$ S.D., n=48, human liver) of ^{3}H-triamcinolone acetonide and 94.3 $\pm$ 2.3% ($\bar{X} \pm$ S.D., n=24, rat liver) and 97.7 $\pm$ 2.4% ($\bar{X} \pm$ S.D., n=24, human liver) of ^{3}H-prednisolone were obtained. The ethanol eluate was dried under a gentle stream of nitrogen at 40°C and the residue was

then dissolved in 250 µl of ethanol-water (30:70 v/v). The sample was now sufficiently clean for injection in the h.p.l.c. chromatograph. For each GCS that was quantified, three standard samples (containing 5.0, 2.0 and 0.5 µM GCS) in denaturated 9000 g supernatant fraction were prepared. The standard samples were extracted as described above.

Analyses: The analyses were performed on a liquid chromatograph from Waters Associates involving two M6000 solvent delivery systems, an M440 UV detector (254 nm), an M710B WISP autoinjector, an M680 automated gradient controller, and a model 3388A integrator system from Hewlett Packard. A column (200x5 mm int. diam.) prepacked with Nucleosil C_{18} (5 µm) (Waters Assoc.) was used as the stationary phase. After 200 µl injections the steroids were eluted with a concave gradient curve (no. 7) with the mobile phase, ethanol-water varying from 30% to 60% ethanol in 45 min). The compounds 16α-hydroxyprednisolone, triamcinolone and fluocinolone were eluted with ethanol-water varying from 30% to 50% ethanol in 20 min. The flow rate was 1 ml/min. Before each injection the system was allowed to equilibrate for 20 min with ethanol-water (30:70 v/v). During the elution, the uv-peaks of the unchanged compound and formed metabolites were recorded. The area under the uv-peaks (AUP) was calculated by the 3388A integrator system and corrected by dividing them by the AUP of the internal standard. A standard curve was

prepared from the standard samples. The correlation coefficient of all standard curves was > 0.999. Identification of metabolites was based on mixing authentic compounds with metabolites from the incubation medium in equal amounts. The mixture was then injected into the h.p.l.c.-system. Positive identification was defined as a proportional increase of the AUP without any peak broadening.

Measurements of radioactivity: Radioactivity was measured by the liquid scintillation technique. The measurements were performed in Instagel^{R} (Packard) using a PA 3255 (Packard) liquid scintillation counter. The counting efficiency was estimated by the external standard channel ratio procedure.

RESULTS

After incubation of the GCS with rat and human liver 9000 g supernatant fraction, the incubation media were extracted on Sep-Pak C_{18} cartridges and then subjected to reversed phase liquid chromatography. The relative retention values of the different GCS after chromatography are given in Table 1 and an example of a uv-chromatogram is shown in Figure 2. Quantification was performed by calculations of the area under the uv-peaks (254 nm). This methodological development thus enabled us to analyse GCS in the liver samples at biological relevant concentrations without the use of radiolabelled compounds as previously described (Andersson et al., 1982b). The analyses of the incubation samples demonstrated a decrease of the area under the unchanged GCS uv-peak (254 nm) simultaneously with the formation of metabolites. Metabolites formed were detected at 254 nm provided that a conjugated double bond system was present in ring A.

The disappearance of four GCS during incubation at the concentration of 5 μM with human liver 9000 g supernatant fraction is shown in Figure 3. Since the disappearance of all GCS seemed to follow first order kinetics, the half-life ($t_{1/2}$) was used to characterize the rate of metabolic degradation of the different GCS (Tables 2 and 3). No degradation of the GCS occurred in denatured supernatants (65°C).

Introduction of a 16α-hydroxy group in the prednisolone molecule was found to slow down the biotransformation rate by a factor of about two in rat liver 9000 g supernatant fraction (Table 2). The substitution of the 16α,17α-hydroxy groups with a symmetric acetal group as in desonide, led to a biotransformation rate between those calculated for prednisolone and 16α-hydroxyprednisolone. However, substitution of the 16α,17α-hydroxy groups with the non-symmetric acetal group, as in budesonide, enhanced the biotransformation rate several-fold in comparison with prednisolone, 16α-hydroxyprednisolone and desonide. The 22R-epimers of the non-symmetric acetals were metabolised about twice as rapidly as the 22S-epimers. Hydrogenation of the 1,2-double bond of prednisolone and the budesonide epimers enhanced the biotransformation rate about 9-fold. Fluoro-substitution of 16α-hydroxyprednisolone and desonide was found to slow down the biotransformation rate marginally. Fluorosubstitution of the budesonide epimers did not seem to affect the biotransformation rate.

The results obtained with human liver 9000 g supernatant fraction (Table 3) differed from those obtained with rat liver 9000 g supernatant fraction in three ways. Firstly, substitution of the 16α,17α-hydroxy groups with the symmetric acetal group as in e.g. desonide enhanced the biotransformation rate more efficiently in human liver

than in rat liver. However, as in rat liver, the effect of the non-symmetric acetal substituent on the biotransformation rate was more pronounced. Secondly, in human liver, the enhancing effect of 1,2-hydrogenation on the biotransformation rate was not as extensive as in rat liver (only about 2-fold in human liver in comparison with a 9-fold increase in rat liver). Thirdly, steroid nucleus fluorination (in 6α- and 9α-positions) of especially desonide and the budesonide epimers, enhanced the biotransformation rate several-fold in human liver. The difluorinated GCS showed the fastest biotransformation rate.

DISCUSSION

The analytical method used in this study was developed to determine the biotransformation rate of non-radiolabelled GCS in liver 9000 g supernatant fraction. The GCS were incubated at a concentration of 5 μM and their disappearance could be followed down to approximately 0.3 μM. Results obtained at this incubation concentration agree with those obtained at 5-50 times lower concentration using radiolabelled budesonide epimers, triamcinolone acetonide and hydrocortisone (Andersson et al., 1982b).

In the present study each compound was in most cases incubated once. In those cases, where the GCS were incubated at two separate occasions, a good agreement in half-life was obtained (Tables 2 and 3). Relative half-lives obtained from an earlier study with radiolabelled compounds (budesonide epimers, triamcinolone acetonide and hydrocortisone) agreed well with data obtained in this study (Andersson et al., 1982b).

Irrespective of whether rat or human liver 9000 g supernatant fraction was used as incubation medium, introduction of the 16α-hydroxy group had a slowing down effect on the biotransformation rate of GCS (Tables 2 and 3). Substitution in the 16α,17α-positions with the symmetric or the non-symmetric acetal group, was found to enhance the biotransformation rate. When both types of acetal compounds

containing the same number of fluorine atoms were compared, it was evident that the non-symmetric acetals (both the 22R- and 22S-epimers) were biotransformed more rapidly than the symmetric acetal. Furthermore, the 22R-epimers of the non-symmetric acetal GCS were more rapidly biotransformed than the 22S-epimers which is in agreement with an earlier study on budesonide (Andersson et al., 1982b). Differences in metabolic pathways between the acetal GCS can partly explain these results. Accordingly, while both types of acetal GCS are biotransformed by 6β-hydroxylation (Kupfer and Partridge, 1970; Teitelbaum et al., 1981 and Edsbäcker et al., 1983), budesonide is also biotransformed via a mono-oxygenation catalysed acetal cleavage to 16α-hydroxyprednisolone, a pathway specific for the 22R-epimer (Edsbäcker et al., 1983). From the h.p.l.c.-retention times of the metabolites formed in the present study, acetal cleavage of the 22R-epimers of 9α-fluorobudesonide and 6α.9α-difluorobudesonide was evident, resulting in formation of triamcinolone and fluocinolone respectively. No such metabolic route was found with the 22S-epimers or with the symmetric acetals which is in agreement with results of Kripalani et al. (1975), Teitelbaum et al. (1981) and Edsbäcker et al. (1983). These findings show that the more rapid biotransformation of at least the 22R-epimers of budesonide-type partly depend on the presence of unique metabolic sites in the non-symmetric acetal moiety. Another contributing factor in favour of the non-symmetric acetals might be a

more suitable lipophilicity, allowing a better binding to oxidative enzymes (e.g. P-450) which are buried in the endoplasmic reticulum (Estabrook et al., 1975).

Some interesting differences between the results from rat and human liver 9000 g supernatant fraction were obtained. Hydrogenation of the budesonide epimers and of prednisolone in the 1,2-positions, enhanced the biotransformation rate about 9-fold in rat liver but only 2-fold in human liver. The enhancing effect is probably explained by enhanced ring-A reduction as was suggested by Brown and Anason (1958). This suggested explanation is supported by the fact that no uv-absorbable (254 nm) metabolites of the hydrogenated GCS (hydrocortisone and the epimers of 1,2-dihydrobudesonide) were detected in the h.p.l.c.-chromatograms after incubation with rat liver. Hydrocortisone is also known to be biotransformed mainly by 3-keto-4-ene reduction and only to a less extent by e.g. 6β-hydroxylation (Seutter, 1975). The quantitative different effect of 1,2-hydrogenation in rat and human liver may be explained by a preference for ring-A reduction in rat liver, compared with human liver.

Steroid nucleus fluorination (in the 9α- and 6α-positions) was also found to affect the biotransformation rate of GCS differently in rat and human liver 9000 g supernatant fraction. In rat liver, fluorination only had a marginal

decreasing effect on the biotransformation rate, whereas in human liver fluorination, especially of desonide and the budesonide epimers, enhanced the biotransformation rate several-fold. As these GCS are biotransformed mainly by mono-oxygenase catalysed reactions (e.g. Kupfer and Partridge, 1970; Teitelbaum et al., 1981; and Edsbäcker et al., 1983) these results with human liver are interpreted as enhanced oxidative metabolism.

Our in vitro data on liver biotransformation correlates well with in vivo data on oral bioavailability. In humans the oral bioavailability of flunisolide (6α-fluorodesonide) was reported to be 20% and in rats close to 100% (Chaplin et al., 1980 and Chu et al., 1979). The figure for budesonide (1:1 epimer mixture) is 10.4 ± 4.3% (humans) and 10-30% (rats) (Ryrfeldt et al., 1982 and Andersson, P. and Ryrfeldt, Å., unpublished observations). Furthermore, unpublished data obtained with humans (Borgå, O. and Tönnesson, M.) show that the oral bioavailability of the 22R-epimer of budesonide is about half (7.8 ± 2.7%) that of the 22S-epimer (18.1 ± 4.9%). The oral bioavailability of the relatively slowly biotransformed prednisolone was reported to be 78-85% in humans (Bergrem et al., 1983). These in vivo data correlate well with our in vitro results in the sense that a rapid liver biotransformation in vitro corresponds with a high first pass metabolism in the liver and consequently low oral bioavailability.

However, it has recently been reported that hydrocortisone showed almost complete oral bioavailability in the rat (Chanoine and Junien, 1984). According to our in vitro data, hydrocortisone is rapidly biotransformed in the rat liver. The reason for this discrepancy is not known. A low oral bioavailability of triamcinolone acetonide (9α-fluorodesonide), fluocinolone acetonide (6α,9α-difluorodesonide) as well as of the fluorinated budesonide compounds in humans may be assumed from our in vitro data. Of these compounds, triamcinolone acetonide (like flunisolide and budesonide) is used clinically for aerosol treatment without causing any significant systemic effects (Williams et al., 1974).

In rat liver preparation, the metabolism of the fluorinated symmetric acetal GCS was found to proceed at a much slower rate than in human liver. As mentioned above, the oral bioavailability of flunisolide (6α-fluorodesonide) in rats is almost complete in comparison with an oral bioavailability of 20% in humans (Chu et al., 1979 and Chaplin et al., 1980). Our results with rat liver show that not only flunisolide but also triamcinolone acetonide (9α-fluorodesonide) and fluocinolone acetonide (6α,9α-difluorodesonide) are slowly biotransformed, suggesting a high oral bioavailability in rats also of these drugs. This is supported by investigations by Brattsand and coworkers (1982a and 1982b), who found that 9α- and

6α-fluoro substitution of desonide elevated the systemic activity after oral treatment of rats more than it raised the topical antiinflammatory potency. Furthermore, compared with the non-symmetric acetals (e.g. budesonide), the systemic activity of the symmetric acetals (e.g. desonide) after oral treatment was exceptionally high. These results are probably caused by a low rate of inactivation of the symmetric acetals during the first pass through the rat liver and consequently a high oral bioavailability. Apparently, data obtained with acetal GCS in rat models should be interpreted with care, especially when extrapolations of animal data to man are considered.

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Table 1. Relative retention values of the different glucocorticosteroids obtained after h.p.l.c.. The stationary phase was Nucleosil C_{18} (5 µm), the mobile phase was ethanol-water with a non-linear gradient from 30:70 to 60:40 (v/v) in 45 min. Flow rate: 1.0 ml/min.

Structural alterations	Rt-value relative to internal standard (D-5086)				
	Prednisolone	16α-Hydroxy-prednisolone	Desonide	Budesonide (22R)	Budesonide (22S)
none	0.50	0.28	0.67	0.85	0.87
1,2-hydrogenation	0.50	-	-	0.87	0.89
9α-fluorination	-	0.25	0.65	0.81	0.84
6α-fluorination	-	-	0.67	-	-
6α,9α-fluorination	-	0.26	0.67	0.82	0.86

Table 2. The effect of structural alterations on the biotransformation rate ($t_{1/2}$) of glucocorticosteroids during incubation with rat liver 9000 g supernatant fraction. Initial glucocorticoid concentration: 5 μM.

Structural alterations	$t_{1/2}$ (min)				
	Prednisolone	16α-Hydroxy-prednisolone	Desonide	Budesonide (22R)	Budesonide (22S)
none	41.4	85.4	67.0	13.6 18.0*	30.3 29.7*
1,2-hydrogenation	4.9 5.0*	-	-	2.0	3.6
9α-fluorination	-	214	117	19.4	28.0
6α-fluorination	-	-	123	-	-
6α,9α-fluorination	-	299	118	15.7	37.2

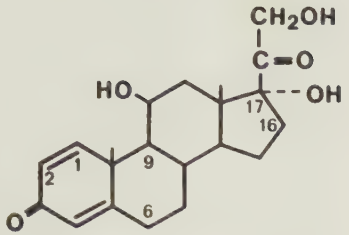
* incubation on separate occasions

Table 3. The effect of structural alterations on the biotransformation rate ($t_{1/2}$) of glucocorticosteroids during incubation with human liver 9000 g supernatant fraction. Initial glucocorticoid concentration: 5 μM.

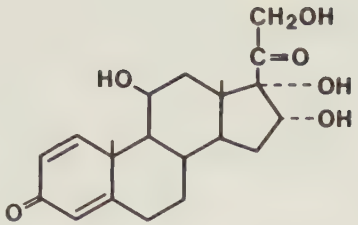
Structural alterations	$t_{1/2}$ (min)				
	Prednisolone	16α-Hydroxy-prednisolone	Desonide	Budesonide (22R)	Budesonide (22S)
none	83.7	n.b.d.	52.5	6.1 5.3*	11.2 10.8*
1,2-hydrogenation	37.2 37.3*	-	-	3.3	6.8
9α-fluorination	-	305	16.2	1.9	3.9
6α-fluorination	-	-	5.8	-	-
6α,9α-fluorination	-	242	2.7	<1	1.6

n.b.d. = no biotransformation detected

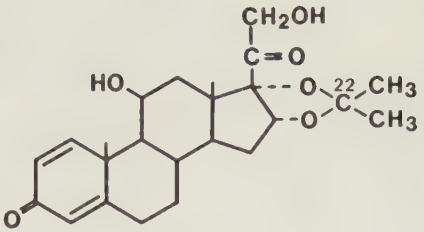
* incubation on separate occasions



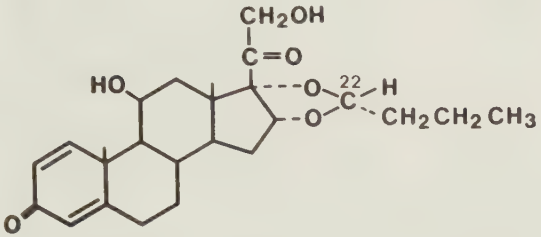
Prednisolone



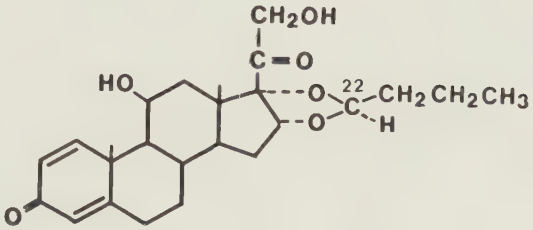
16 α–Hydroxyprednisolone



Desonide



Budesonide (22R)



Budesonide (22S)

Fig. 1

Structural formulas

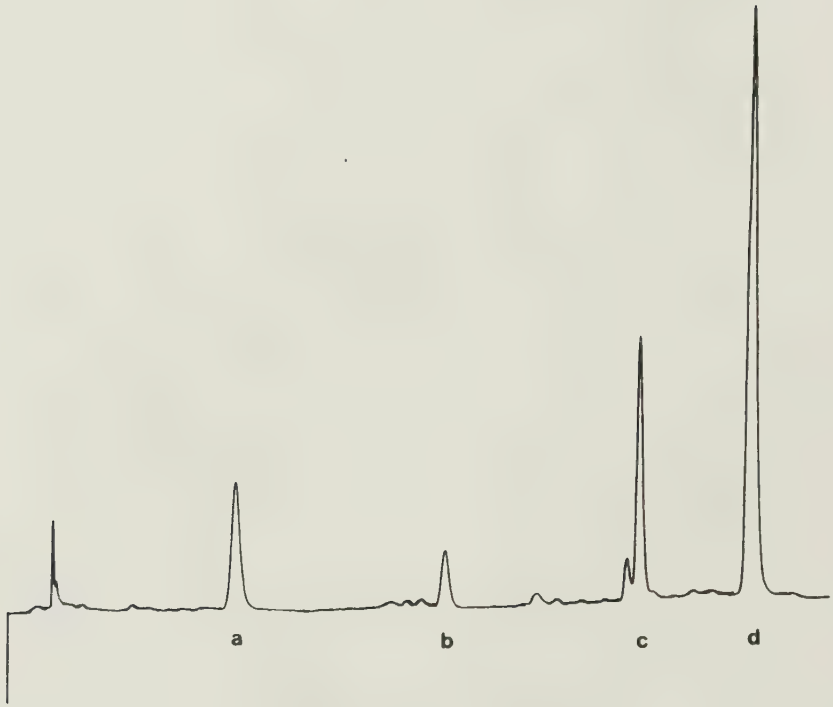


Fig. 2

Uv-chromatogram (254 nm) obtained from h.p.l.c. of a human liver 9000 g sample, incubated for 10 min with the 22R-epimer of budesonide.

Stationary phase: Nucleosil C_{18} (5 µm), mobile phase: ethanol-water concave gradient from 30:70 to 60:40 (v/v) in 45 min, flow rate: 1.0 ml/min. a = 16α-hydroxyprednisolone, b = 22R-epimer of 6β-hydroxybudesonide, c = 22R-epimer of budesonide, d = internal standard.

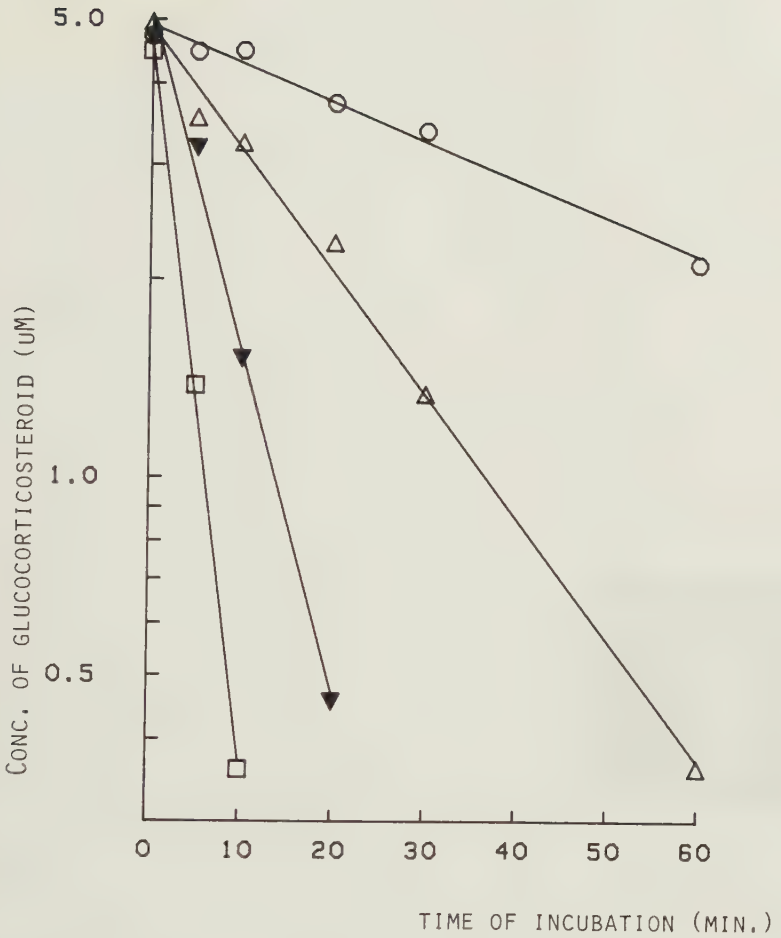


Fig. 3

The disappearance of some glucocorticosteroids during incubation with human liver 9000 g supernatant fraction. o = desonide; △ = triamcinolone acetonide (9α-fluorodesonide); ▼ = flunisolide (6α-fluorodesonide); □ = fluocinolone acetonide (6α,9α-difluorodesonide)

IV

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Biotransformation of the topical glucocorticoids budesonide and beclomethasone 17α,21-dipropionate in human liver and lung homogenate

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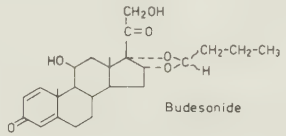
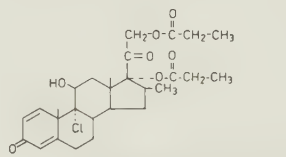
Tritiated budesonide and beclomethasone 17α,21-dipropionate (BDP) were incubated with the 9000*g* supernatant of human liver homogenate. BDP was immediately hydrolysed to beclomethasone 17α-propionate (BMP). BMP was then further biotransformed to polar metabolites. Budesonide was rapidly biotransformed (2–4 times more rapidly than BMP) to metabolites of low glucocorticoid potency. The compounds were also incubated with the 1000*g* supernatant of human lung homogenate. BDP was rapidly hydrolysed to BMP and then more slowly to beclomethasone. Budesonide was not biotransformed in the lung.

The glucocorticoids budesonide I and beclomethasone 17α,21-dipropionate (BDP, II) are both used in the topical treatment (inhalation) of respiratory disorders such as asthma and rhinitis (Hansen & Mygind 1974; Toogood et al 1977; Ellul-Micallef et al 1980; Balle 1982). As judged from vasoconstriction data budesonide is 2–3 times more potent than BDP (Johansson et al 1982), which indicates that it is clinically more potent than BDP. However, budesonide is significantly less potent that BDP in depressing plasma cortisol and changing the total or differential white blood cell count especially after oral administration but also after inhalation (Johansson et al 1982). This difference may depend on a more efficient first pass metabolism of budesonide giving metabolites with low biological activity. We describe the in-vitro biotransformation of budesonide and BDP in human liver and lung.





Methods

Tritiated [1,2-^{3}H]budesonide and [1,2-^{3}H]beclomethasone 17α,21-dipropionate ([1,2-^{3}H]BDP) were obtained from the Radiochemical Centre, Amersham, England. The specific activity was 76·6 mCi mg^{-1} [^{3}H]budesonide and 19·0 mCi mg^{-1} [^{3}H]BDP. Hplc analysis using a Nucleosil C_{18} (5 μM) column and ethanol–water as mobile phase (45 : 55 for [^{3}H]budesonide and 50 : 50 for [^{3}H]BDP) showed a radiochemical purity higher than 95%. Non-labelled budesonide, beclomethasone 17α-propionate (BMP), beclomethasone 21-propionate and beclomethasone were supplied by Dr A. Thalén, AB Draco, Sweden. Non-labelled BDP was obtained from LARK SpA, Milan, Italy. The water used was Millipore filtered and dichloromethane, chloroform, heptane and ethanol were of spectroscopic grade. D-Glucose-6-phosphate, NADP and glucose-6-phosphate dehydrogenase were obtained from Sigma, Sweden.

Four human liver samples (samples: 13, 16, 17 and 18) were supplied from the liver bank at Huddinge Hospital, Sweden (von Bahr et al 1980). The sex and age of the donors were F53, M52, M24 and F59, respectively. Each sample was homogenized in ice cold buffer (0·1 mol litre^{-1} K_2HPO_4, KH_2PO_4, pH 7·4, also containing 0·2 mol litre^{-1} of sucrose) with a Polytron PT20 homogenizer for 20–30 s. The homogenates were centrifuged at 9000*g*. The protein concentration was estimated according to Lowry et al (1951) and diluted to 1 mg ml^{-1}. Three human lung samples were obtained from lobectomy patients at the University Hospital of Lund, Sweden. The age of the donors was 51, 58 and 75 years. The lungs were pooled and homogenized as described above. The homogenate was centrifuged at 1000*g*. The protein concentration was measured and diluted to 4 mg ml^{-1}.

[^{3}H]Budesonide and [^{3}H]BDP were incubated at a concentration of 0·1 μmol litre^{-1} with the 9000*g* liver supernatants and with the 1000*g* lung supernatant at 37 °C in the presence of carbogen gas (95% O_2, 5% CO_2). Each incubation (volume 6 ml) also contained 6 mg of protein (liver) or 24 mg of protein (lung), 60 μmol of $MgCl_2$, 45 μmol of glucose-6-phosphate, 1·8 μmol of NADP, and 2·5 units of glucose-6-phosphate dehydrogenase. Blank incubations using denatured (boiled) supernatants were also performed.

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After an equilibrium period for 10 min at 37 °C, the incubations were started by the addition of 60 µl ethanol containing either [^{3}H]budesonide or [^{3}H]BDP. One human serum sample (diluted 1 : 1 with buffer) was also incubated with [^{3}H]budesonide and [^{3}H]BDP. Aliquots (0·5 ml) were taken from each incubation flask and transferred into borosilicate test tubes containing either non-labelled budesonide (60 µg) or BDP, BMP and beclomethasone (71, 63 and 56 µg, respectively) as internal standard in 0·5 ml ethanol. The samples were then frozen on dry ice (−60 °C). To each sample, 4·0 ml of dichloromethane was added. Extraction was by gentle shaking for 30 min, followed by centrifugation for 15 min at 2000 rev min^{-1}. The organic phase (3·0 ml) was collected and taken to dryness under a gentle stream of nitrogen. The residue was then dissolved in 200 µl of the mobile phase used in hplc. By extraction of denatured incubation samples from liver, a recovery of 89·5% (s.d.: 0·8%, n = 5, [^{3}H]budesonide) and 101·8% (s.d.: 5·6%, n = 5, [^{3}H]BDP) of the total radioactivity were obtained.

The analyses of [^{3}H]budesonide and [^{3}H]BDP–BMP were by hplc (Waters): stationary phase Nucleosil C_{18} (5 µM) (Column: 5 × 200 mm), mobile phase ethanol–water (45 : 55 and 50 : 50, respectively), flow rate 1·0 ml min^{-1}. After injection (100 µl) the effluent was collected into scintillation vials and the tritiated peaks were assayed for radioactivity. The uv-peaks of the internal standards were recorded by a uv-detector and the ratio between radioactivity and area under uv-peak was calculated. At 0 min of incubation, the ratio was considered to reflect a relative concentration of 1. [^{3}H]Budesonide, [^{3}H]BDP, [^{3}H]BMP and [^{3}H]beclomethazone were identified on the basis of their retention times compared with those of the internal standards.

Results and discussion

During addition of [^{3}H]BDP to the 9000*g* liver supernatants, the drug was immediately biotransformed to a metabolite having a similar hplc retention time as beclomethasone 17α-propionate or beclomethasone 21-propionate. The identity of the metabolite was checked by gel chromatography on a Lipidex 5000 (Packard) column with heptane–chloroform (1 : 1) as mobile phase. In this system the 17α- and 21-propionate esters of beclomethasone separated. All radioactivity co-eluted with beclomethasone 17α-propionate (BMP). Since the vasoconstriction potency of BMP is almost as high as that for BDP, the hydrolysis of BDP to BMP does not represent an inactivation step (Harris 1975). To be deactivated, BMP has to be hydrolysed to beclomethasone and/or biotransformed by oxidative or reductive pathways to polar metabolites. The relative biotransformation rate of [^{3}H]budesonide and [^{3}H]BMP in homogenates from the human livers followed a pattern shown in Fig. 1. The [^{3}H]budesonide was biotransformed 2–4 times more rapidly than

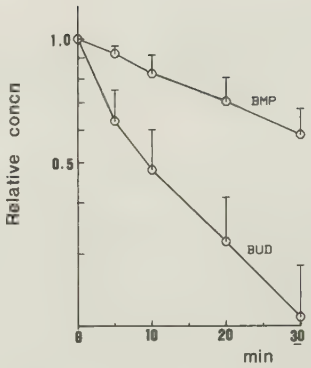

FIG. 1. Relative concentrations of unchanged budesonide (BUD) and beclomethasone 17α-propionate (BMP) during incubation of BUD and beclomethasone 17α,21-dipropionate (BDP) in four human liver 9000*g* supernatants. Initial steroid concentration: 0·1 µmol litre^{-1}. The data points are expressed as mean ± s.e.m. Part of this figure has been published in Eur. J. Resp. Dis. 1982, 63 (122): 86–95.

[^{3}H]BMP. While [^{3}H]budesonide was biotransformed to the relative inactive metabolites 6β-hydroxybudesonide and 16α-hydroxyprednisolone (Edsbäcker et al 1983; Dahlberg et al 1984). [^{3}H]BMP was biotransformed to unknown metabolites. None of these was found to co-elute with beclomethasone during hplc analyses. No biotransformation occurred when incubating [^{3}H]budesonide and [^{3}H]BDP with denatured incubation medium.

During incubation with the 1000*g* lung supernatant, [^{3}H]BDP was rapidly hydrolysed to [^{3}H]BMP (Fig. 2). After 5 min only18% of the radioactivity was attributed to [^{3}H]BDP and the rest to [^{3}H]BMP, which then was very slowly biotransformed to a compound with the same retention time as beclomethasone.

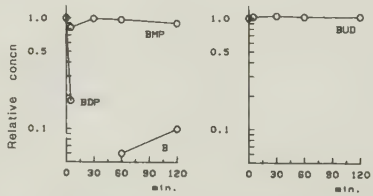

FIG. 2. Relative concentration of unchanged beclomethasone 17α,21-dipropionate (BDP), beclomethasone 17α-propionate (BMP) and beclomethasone (B) and of unchanged budesonide (BUD) during incubation of BDP and BUD with the human lung 1000*g* supernatant. Initial steroid concentration: 0·1 µmol litre^{-1}.

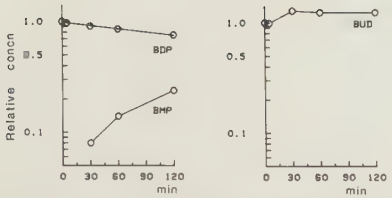

FIG. 3. Relative concentration of unchanged beclomethasone 17α,21-dipropionate (BDP) and beclomethasone 17α-propionate (BMP) and of unchanged budesonide (BUD) during incubation of BDP and BUD with human serum (diluted 1:1 with buffer). Initial steroid concentration: 0·1 μmol litre^{-1}.

The results are in agreement with those reported by Martin et al (1974) and Ronca-Tesloni (1983) *and* are due to a relatively high activity of esterase enzymes in the lung. They also verify that an ester group in the 21-position is more easily hydrolysed than that in the 17α-position (O'Neill & Carless 1980). Budesonide, on the other hand, was not biotransformed in the lung (Fig. 2). Since budesonide in the liver is biotransformed mainly by oxidative and to some extent also reductive pathways, the results indicate a very low activity of these enzymes in the lung.

The two drugs were also incubated with one human serum sample (diluted 1:1 with buffer). [^{3}H]BDP was slowly hydrolysed to [^{3}H]BMP (Fig. 3). At 2 h of incubation about 76% of the radioactivity was attributed to unchanged [^{3}H]BDP and the rest to [^{3}H]BMP. No [^{3}H]beclomethasone could be detected. [^{3}H]budesonide was not biotransformed in serum (Fig. 3).

The present data help to elucidate the pharmacodynamics of the two glucocorticoids. After topical deposition in the respiratory tract, both drugs are systemically absorbed, probably with most of their biological activity preserved. However, in topical (aerosol) therapy, the major fraction of the discharged dose is swallowed and then absorbed from the gastrointestinal tract (Newman et al 1980; Davies 1982). The absorbed drug can induce systemic side effects. To minimize these effects it is essential that an efficient first pass inactivation in the liver takes place. For steroid esters, inactivation by hydrolysis in the lumen and/or mucosa of the small intestine can also be of importance. In this study, [^{3}H]budesonide was found to be more rapidly inactivated by biotransformation than [^{3}H]BDP in human liver homogenate. The efficient liver biotransformation of budesonide is also reflected by a low oral availability (10·7 ± 4·3%) as was shown by Ryrfeldt et al (1982) in healthy volunteers. These results may partly explain the relatively lower systemic effects found with budesonide compared with BDP at higher clinical dosages.

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V

METABOLIC ACETAL SPLITTING OF BUDESONIDE – A NOVEL INACTIVATION PATHWAY FOR TOPICAL GLUCOCORTICOIDS.

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Running title: METABOLIC ACETAL SPLITTING OF BUDESONIDE

ABSTRACT:

Topical glucocorticoids usually have a high intrinsic glucocorticoid potency and may after systemic uptake induce side effects. The systemic inactivation of budesonide is rapid due to extensive liver biotransformation. The major metabolic pathway, 16α,17α-acetal splitting, is unique for budesonide within this group of compounds. This biotransformation is catalyzed by microsomal monooxygenases and proceeds via hydroxylation and subsequent rearrangement to an intermediary ester. The ester is cleaved by hydrolysis to 16α-hydroxyprednisolone and butyric acid. The hydrolysis product 16α-hydroxyprednisolone has strongly reduced glucocorticoid activity.

Budesonide (fig. 1) is a potent glucocorticoid, used clinically in the local treatment of skin (1) and respiratory diseases of an inflammatory and/or allergic ethiology (2). The drug deviates structurally from the common topical 16α,17α-acetonide glucocorticoids by a new type of nonsymmetric 16α,17α-acetal substituent with a 1:1 ratio between epimers 22R and 22S (3). This chemical modification not only improves the topical anti-inflammatory potency at least five-fold as compared with the symmetric 16α,17α-acetonide analogue (4), but also provides additional sites for metabolic inactivation in the liver (5,6). The major budesonide metabolite, both in vitro in human liver preparations[1] (5,6) and in plasma after i.v. administration to healthy subjects[1], is 16α-hydroxyprednisolone (fig. 1). The glucocorticoid potency of this metabolite is less than 1% of that of the parent compound (7). In the pharmacological and toxicological evaluation of new chemical entities, the metabolism is always of importance. Experiments were therefore undertaken to in more detail elucidate the mechanism of the metabolic 16α,17α-acetal splitting, which involves only the 22R-epimer of budesonide (5,6). This unique and highly efficient inactivation pathway contributes in a fundamental way to the favourable ratio between local therapeutic effect and systemic side effects noted for the drug (8,9).

Chemicals. Budesonide ((22RS)-16α,17α-butylidenedioxy-11β,21-dihydroxypregna-1,4-diene-3,20-dione) and ($^{2}H_{8}$)budesonide ((22RS)-(22,23,23,24,24,25,25,25-$^{2}H_{8}$)-16α,17α-butylidenedioxy-11β,21-dihydroxypregna-1,4-diene-3,20-dione, were prepared and resolved into their 22R- and 22S-epimers as previously described (3,10).

16α-Butyryloxyprednisolone. To a suspension of 0.75 g of 16α-hydroxyprednisolone in dry dioxane (7.5 ml) was added trimethylorthobutyrate (0.75 ml), anhydrous methanol (6 drops) and 70% of perchloric acid (2 drops). After 5 min the reaction mixture was essentially clear and filtered into a flask containing 0.75 ml of pyridine. Water (25 ml) was added followed by solid NaCl to saturation. The upper phase was separated, dissolved in methylene chloride and dried over Na_2SO_4. The solvents were evaporated and the residue precipitated from methylene chloride-petroleum ether leaving 0.80 g of 16α,17α-(1-methoxy)butylidenedioxy-11β,21-dihydroxypregna-1,4-diene-3,20-dione.

To a solution of this compound in 40 ml of methanol was added 6.5 ml of 2M HCl. After 5 min at room temperature, the reaction mixture was neutralized with $NaHCO_3$ and evaporated. Water (50 ml) was added and the solution was extracted with ethyl acetate, dried and evaporated. The residue was purified by chromatography on a Sephadex LH-20 column, using chloroform as

mobile phase, yielding 0.41 g of a solid product after precipitation from methylene chloride-petroleum ether. The purity as estimated by HPLC (μBondapak C_{18}; 45% ethanol (v/v) in water) was 97.7%. MS (fig. 3C): m/z 447, MH^+; 429, MH^+-H_2O; 387, $MH^+-HCOCH_2OH$; 369, $MH^+-HCOCH_2OH-H_2O$; 359, $MH^+-CH_3(CH_2)_2COOH$; 341, $MH^+-CH_3(CH_2)_2COOH-H_2O$; 329, $MH^+-CH_3(CH_2)_2COOH-HCHO$. ^{13}C-NMR (DMSO-$^2H_6$): δ (ppm) 65.9 (C-21), 74.7 (C-16) and 87.7 (C-17). A comparison with the corresponding carbon resonances of 16α-hydroxyprednisolone, 66.4, 71.2 and 87.5 ppm, respectively, shows a deshielding of only the C-16 atom in the product of a magnitude which is in agreement with acylation of the 16α-hydroxy group (11).

The downfield shift in ^1H-NMR (C^2HCl_3) of the C-16 proton, from δ 4.90 in 16α-hydroxyprednisolone to δ 5.71 ppm in the product and to 5.72 in 16α,21-diacetoxy-11β,17α-dihydroxypregna-1,4-diene-3,20-dione, further confirms the structure of the product as 16α-butyryloxyprednisolone.

NMR spectra were recorded on a Jeol FX 90Q spectrometer (Jeol LTD, Tokyo, Japan) operating in the Fourier-transform mode at a freqency of 89.6 MHz (^1H-NMR) and 22.5 MHz (^{13}C-NMR). Tetramethylsilane and chloroform (δ 77.1 ppm), respectively, were used as internal references.

[1,2-^3H]Budesonide (specific activity 43 Ci/mmol, [22,23,24,25-^3H]budesonide (68 Ci/mmol), [1,2,3,4-^3H]butyraldehyde (50 Ci/mmol), [1-^{14}C]butyric acid (55 mCi/mmol) and ^{18}O-gas (95

atom %) were obtained from The Radiochemical Centre, Amersham, England. The tritium labeled epimers of budesonide were separated and purified (6). The radiochemical purity, determined by HPLC, was >93% in both cases.

A solution of 2,4-dinitrophenylhydrazine (2,4-DNPH, Merck, Darmstadt, Germany) in 2 M HCl was prepared and purified according to Fung et al. (10). Phenylmethylsulfonylfluoride (PMSF), bis(p-nitrophenyl)phosphate (BPNPP), D-glucose-6-phosphate, $NADP^+$, glucose-6-phosphate dehydrogenase and carboxylic ester hydrolase from porcine liver were obtained from Sigma, Sweden. Trimethylsilylimidazole (TMSI) was obtained from Merck. Diethyl ether, ethanol and methanol were of analytical grade. Butyraldehyde was freshly distilled before use. Water was filtrated through a Millipore Super Q 4 module system. All HPLC solvents were helium-degassed prior to use. A 0.1 M potassium phosphate buffer solution, pH 7.4, containing 0.2 M sucrose was used in all incubation experiments.

Incubation experiments. Liver 9000g supernatant fraction was prepared as described previously (6). Livers from a male human donor (age 65 (6)), male Sprague Dawley CD Charles River rats (Charles River Breeding Laboratories, Inc., St. Wilmington, MA) and NMRI-mice (Anticimex, Stockholm, Sweden) were used. Incubations were performed at a liver protein concentration of 3 mg/ml in the presence of $MgCl_2$ (10 mM), glucose-6-phosphate (40 mM), $NADP^+$ (1.8 mM), and glucose-6-phosphate dehydrogenase (1.2 units/ml). The incubation media were gently shaken at 37°C

under either carbogen, ^{16}O- or ^{18}O-atmosphere. The technique for ^{18}O-incubations will be described elsewhere[2].

(22R)-[22,23,24,25-^{3}H]Budesonide (5 μM) and [^{3}H]butyraldehyde (5 μM) were separately incubated with liver preparations up to 30 min, and samples (1 ml) taken and immediately frozen on dry ice. To each sample was added 1 ml of 2,4-DNPH solution (see bove). After thawing and mixing, the samples were allowed to stand at 4°C for 1 h. One millilitre of 75% (v/v) methanol in water was added, and after 30 min the samples were centrifuged at 1300g for 15 min. The supernatant fractions were filtered through Millipore HV disposable filters (pore size 0.45 μm) and samples of 500 μl injected on the HPLC column (see below).

For the identification of butyric acid, a mixture of the (22R)-budesonide (45μg, 0.1 μmol) and ($^{2}H_{8}$)budesonide (45 μg) was incubated with liver 9000g supernatant fraction (10 ml). After incubation the liver homogenate was deproteinized by addition of an equal volume of 2 M HCl and centrifuged at 2200g for 20 min. About 7 ml of the supernatant fraction was saturated with NaCl and extracted with 5 ml of diethyl ether. The sample was centrifuged at 1500g for 5 min and the ether extract dried with Na_2SO_4. The ether was evaporated to dryness under a stream of nitrogen at room temperature and the residue silylated with 20 μl of TMSI. About 3 μl was used for analysis by gas chromatography mass spectrometry (GC-MS).

In one experiment, a mixture of (22R)-budesonide (225 μg, 0.5

μmol), (22R)-($^{2}H_{8}$)budesonide (225 μg) and (22R)-[1,2-^{3}H]budesonide (0.1 μg, 10 μCi) was incubated for 30 min with mouse liver 9000g supernatant fraction (50 ml) either in the absence or presence of esterase inhibitors (PMSF, 1 mM and BPNPP, 5 mM).

Prior to liquid chromatography mass spectrometry (LC-MS) analysis, samples were extracted on Sep-pak C_{18} cartridges (6). After incubation in the presence of esterase inhibitors, part of the extracted sample was separated by HPLC and radioactive peaks collected.

HPLC. The chromatographic equipment consisted of an automated injector (WISP model 710 B), a model 680 gradient controller, two M 6000 pumps, and a M 440 UV-detector (λ = 254 nm) from Waters Associates (Milford, MA). UV-data were processed on a HP 3388 A integrator (Hewlett Packard, Avondale, PA). Radioactivity was detected on line with a Packard Trace 7140 (Packard, Downers Grove, Ill). Separation was performed on a 200 x 5 mm Nucleosil C_{18} 5 μm column (Macherey-Nagel, Düren, Germany). Spectrophotometric data were processed on a HP 3388 A integrator (Hewlett Packard, Avondale, PA). Butyric acid was detected at 210 nm and the 2,4-DNPH-derivative of butyraldehyde at 365 nm. In some experiments a refractive index detector was used to detect butanol. After 10 min of isocratic elution at 1 ml/min with 25% (v/v) methanol in 0.01 M phosphate buffer (pH 3.5), a gradient was run to 97.5% methanol in the same buffer during 50 min.

Mass spectrometric analysis. Mass spectra were obtained using a Finnigan (San José, California) 4500 instrument interfaced to a Finnigan INCOS data system.

Butyric acid was gas chromatographed and identified as its trimethylsilyl ester after reaction with TMSI according to ref. 13. The gas chromatographic column (180 cm x 2 mm I.D.) was packed with 3% OV-1 on Chromosorb, 60-80 mesh. It was operated at 40°C for 1 min after injection, and then the temperature was raised to 100°C at a rate of 5°C/min. The injector temperature was 100°C. Helium was used as carrier gas at a flow rate of 10 ml/min. Chemical ionization mass spectra were obtained using methane as reagent gas at an indicated ion source pressure of 0.4-0.5 torr. Data acqusition was started 2.5 min after injection and spectra were repetitively recorded between m/z 130 and 180.

A moving belt LC-MS interface (Finnigan MAT) with a polyimide belt was used for the on line LC-MS investigations. The methodology was identical to that used previously (6) and will be described in detail elsewhere[2].

Results and discussion

The metabolic acetal splitting of budesonide giving 16α-hydroxyprednisolone requires NADPH. It is furthermore catalyzed by liver microsomal enzymes and inhibited by SKF 525A (5). Liver monooxygenases generally have these features, but an oxidative reaction cannot in a simple way explain the molecular events of the biotransformation. Budesonide, being chemically stable against hydrolysis (unpublished observations), may theoretically be hydrolyzed enzymatically to 16α-hydroxyprednisolone and butyraldehyde.

To elucidate whether butyraldehyde was formed during the acetal cleavage, [22,23,24,25-^{3}H]budesonide was incubated for 30 min at 37°C with liver 9000*g* supernatant fraction from rat, mouse and man in the presence of NADPH. In these experiments, using derivatization with 2,4-DNPH (12) and HPLC separation, no tritium labeled butyraldehyde could be detected. Instead a radioactive compound tentatively identified as butyric acid was formed.The formation of butyric acid was confirmed by incubating a mixture of budesonide and ($^{2}H_{8}$)budesonide with liver preparations from rat, mouse and man. The trimethylsilyl derivatives of unlabeled and deuterium labeled butyric acid were identified by GC-MS in all investigated species. The possibility of rapid conversion of butyraldehyde to butyric acid was excluded, since only tritum labeled butanol could be detected after incubation of [^{3}H]butyraldehyde with the same liver preparations. We therefore conclude that the formation of

butyric acid is an integral part of the metabolic 16α,17α-acetal side chain splitting of budesonide.

When the (22R)-epimer of [1,2-^{3}H]budesonide was incubated with mouse liver 9000g supernatant fraction for 30 min, two metabolites could be detected (fig. 2A). These have previously been identified as 16α-hydroxyprednisolone and 6β-hydroxybudesonide (5). However, if esterase inhibitors were added prior to incubation, the formation of 16α-hydroxyprednisolone was inhibited. Instead a new peak appeared in the chromatogram (fig. 2B). When the compound corresponding to this peak, was isolated and incubated in buffer with porcine liver carboxylic ester hydrolase for 5 min at 37°C, the compound was completely hydrolyzed to 16α-hydroxyprednisolone. These results indicate that the 16α,17α-acetal splitting of budesonide proceeds via a previously unknown intermediary ester of 16α-hydroxyprednisolone, which in the presence of liver esterase activity instantaneously is hydrolyzed to 16α-hydroxyprednisolone and butyric acid.

To elucidate the structure and mode of formation of this intermediary ester, an incubation with mouse liver 9000g supernatant fraction was performed in the presence of esterase inhibitors, using the 22R-epimer of budesonide and ($^{2}H_{8}$)budesonide. A sample from this incubation was analyzed by LC-MS. The chemical ionization mass spectrum of the intermediary compound (fig. 3A) showed peaks at m/z 447 and 454, which represent the protonated molecular ions expected after an oxidation of budesonide and

($^{2}H_{8}$)budesonide in the 16α,17α-acetal group. The difference of 7 u between the MH^{+} ions of the unlabeled and deuterium labeled material showed that one of the eight deuterium atoms of the acetal moiety was lost. When a similar incubation was performed under ^{18}O-atmosphere, ^{18}O was incorporated in the intermediary compound (fig. 3B). The ions at m/z 389 and 396, remaining after loss of the 17β side chain (MH^{+}-$CHOCH_2OH$), also contained ^{18}O (c.f. figs 3A and 3B). The ion at m/z 359, remaining after loss of butyric acid from the protonated molecular ion, did, however, not contain ^{18}O. In a similar incubation performed under ^{18}O atmosphere but in the presence of esterase activity, ^{18}O was not incorporated in 16α-hydroxyprednisolone. No ester intermediate was detected in this experiment. Taken together, these findings show that ^{18}O must have been incorporated in the acetal group, resulting in the formation of the intermediary butyrate ester. The ions at m/z 429 and 436 in Fig. 3A are formed by loss of water from the MH^{+} ions. The corresponding ions in Fig. 3B were also found at m/z 429 and 436, indicating loss of $H_2{}^{18}O$. A mechanism has been proposed for the loss of water from the molecular ion of ethyl acetate (14). Although one should be careful to extrapolate results from electron impact ionization to chemical ionization, the proposed mechanism offers an attractive explanation for the loss of $H_2{}^{18}O$ from the ester intermediate (scheme 1).This mechanism is plausible only if the butyrate ester is attached in the 16-position and not in the 17-position, because it requires a CH_2-group next to the alcohol oxygen of the ester group. The mechanism furthermore requires the ^{18}O-atom to be bound as acyl

oxygen at the 22-carbon. Comparison with authentic 16α-butyryl-oxyprednisolone showed that the HPLC retention time was identical to that of the ester intermediate and that their mass spectra showed the same characteristic ions (fig 3C). The results presented above strongly indicate that the ester intermediate is 16α-butyryloxyprednisolone.

We have in this study presented data concerning the metabolic 16α,17α-acetal splitting of budesonide, which are in accordance with the mechanism presented in fig. 4. Atmospheric oxygen will be incorporated at the 22-carbon of budesonide by a microsomal monooxygenase, i.e. cytochrome P-450. The resulting hypothetical product, 16α-hydroxyprednisolone-16α,17α-hemiorthobutyrate, is expected to be chemically labile and will rearrange to the energetically more favourable 16α-butyryloxyprednisolone isomer. Similar labile hemiorthoester intermediates are suggested to be formed in the acyl migration of glucocorticoid 17- and 21-esters (15,16). In the presence of liver esterase activity, 16α-butyryloxyprednisolone is instantaneously hydrolyzed to 16α-hydroxyprednisolone and butyric acid. The high capacity of liver esterases may explain why the intermediary ester previously has not been detected in liver homogenates (5) or plasma[1] after budesonide administration. The same mechanism as presented in fig. 4, has been suggested in the cytochrome P-450-catalyzed oxidation of methylene--dioxyphenyl compounds (1,3-benzodioxoles) (17). In that study, however, only the end products were identified.

The currently used symmetric 16α,17α-acetonide glucocorticoids have a metabolically stable dioxolane group (5,18,19). The dioxolane 22-carbon of these compounds provides no site for hydroxylation, probably due to the lack of a hydrogen atom at this carbon. We suggest here, that the dioxolane group of a new topical glucocorticoid, budesonide, is submitted to extensive biotransformation by a mechanism, novel for 16,17-substituted glucocorticoids. The acetal splitting of budesonide increases the overall rate of inactivation and hereby reduces the risk for systemic side effects.

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Footnotes:

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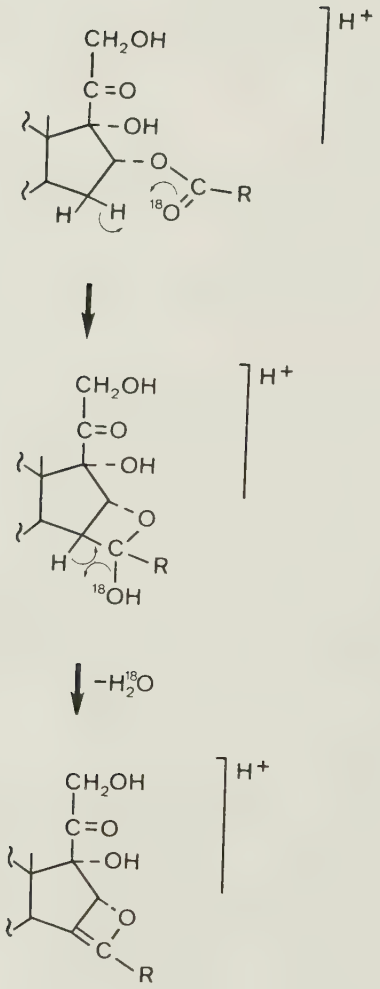
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C=O
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-O
C
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18O
H+
CH2OH
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CH2OH
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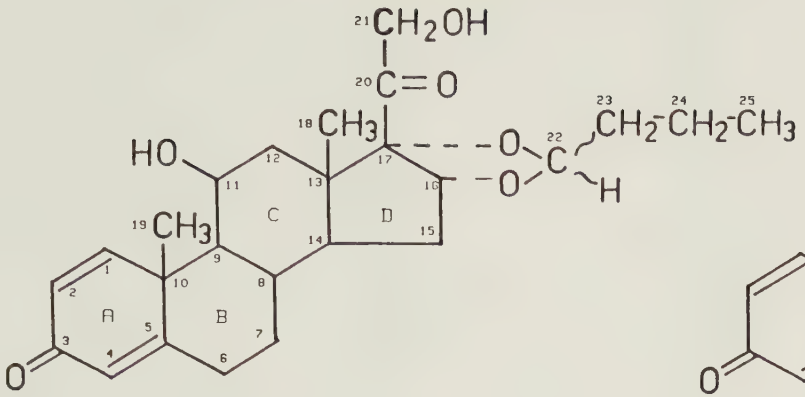
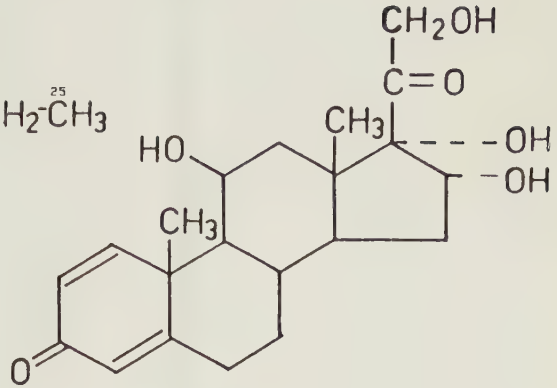
Scheme 1.



Budesonide



16α-Hydroxyprednisolone

FIG. 1. Chemical structure of budesonide and its major metabolite, 16α-hydroxyprednisolone.

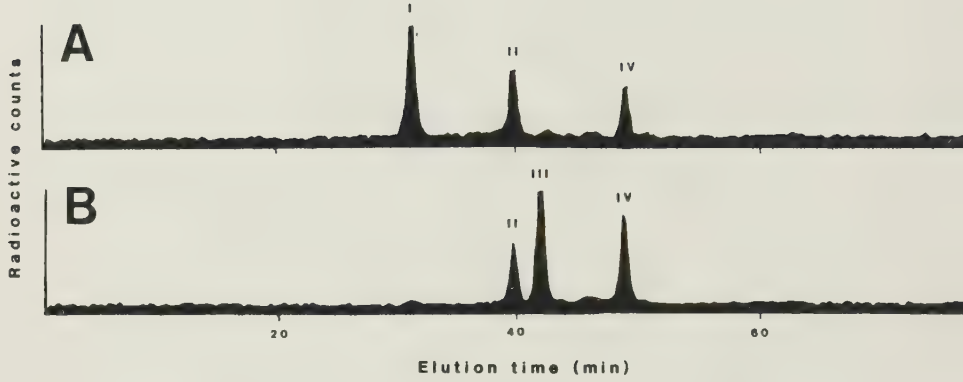


FIG. 2. Radiochromatograms obtained after incubation of (22R)-[1,2-^{3}H]budesonide with mouse liver 9000g supernatant fraction in the absence (A) and presence (B) of esterase inhibitors.

The separated compounds correspond to 16α-hydroxyprednisolone (I), 6β-hydroxybudesonide (II), (22R)-budesonide (IV) and the intermediary compound (III). After extraction by Sep-pak C_{18}, separation was achieved on a Nucleosil C_{18} column using a gradient of methanol in 0.01 M phosphate buffer as mobile phase at a flow rate of 1 ml/min.

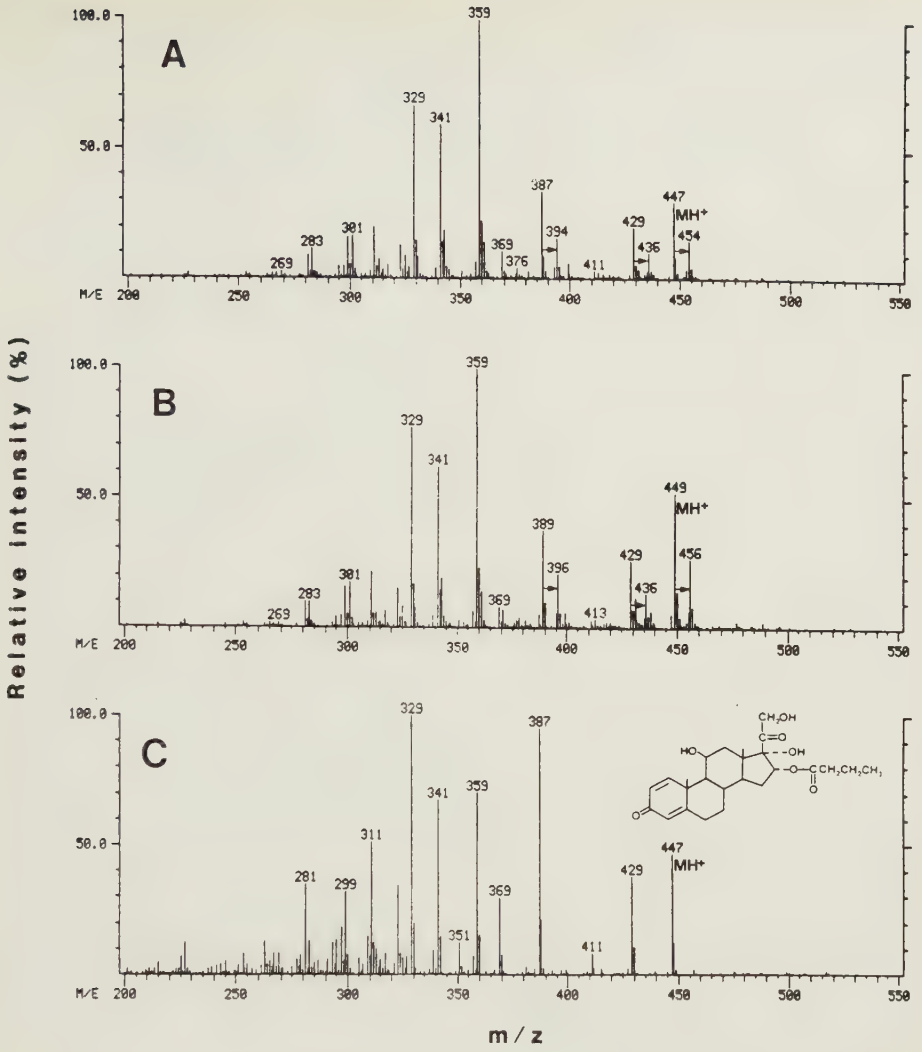


FIG. 3. Chemical ionization (CH_4) mass spectra of **A**; the intermediary ester obtained after incubation in an atmosphere containing ^{16}O, **B**; the intermediary ester obtained after incubation in an atmoshere containing ^{18}O and **C**; authentic 16α-butyryloxyprednisolone.

Arrows indicate deuterium containing ions. The incubations of the (22R)-epimer of budesonide and ($^2H_8$)budesonide were performed with mouse liver 9000g supernatant fraction in the presence of esterase inhibitors. After Sep-pak C_{18} extraction, LC-MS was run as described previously (6).

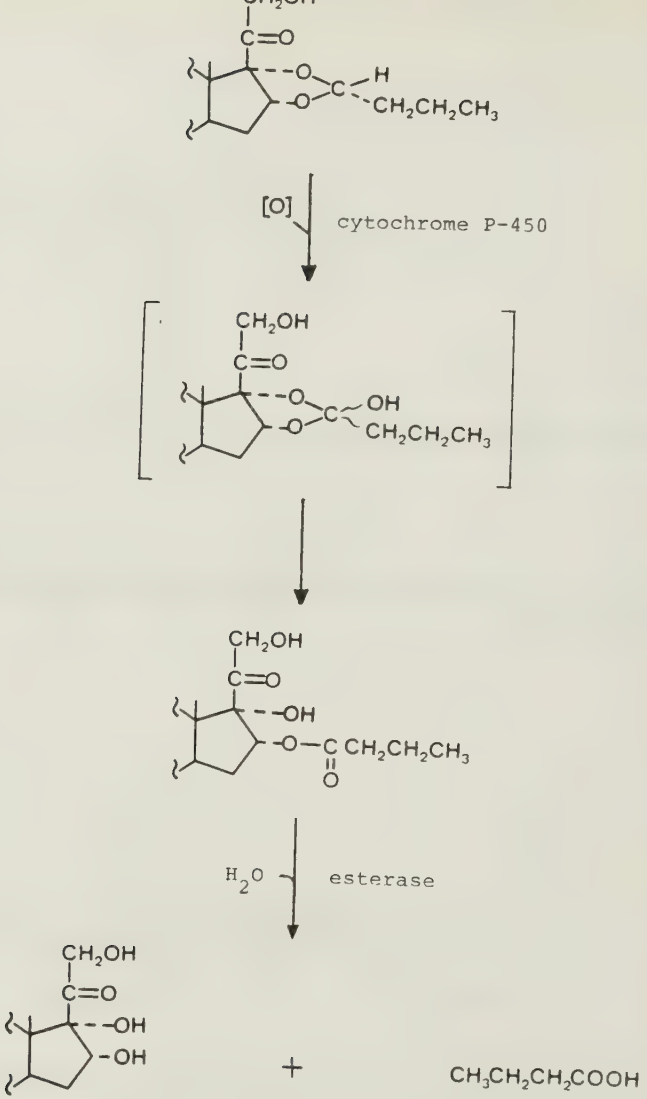


FIG.4. Suggested mechanism for the 16α,17α-acetal splitting of (22R)-budesonide.

Structure in brackets denote a postulated hemiorthoester intermediate.

VI

Title: TISSUE DISTRIBUTION AND FATE OF BUDESONIDE IN THE MOUSE

Running title: Tissue distribution of budesonide

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Key words: Pharmacokinetics - autoradiography - - distribution - biotransformation - mice - - budesonide

ABSTRACT

The tissue distribution and pharmacokinetics of ^{3}H-budesonide were studied in the mouse after intravenous administration. The drug was rapidly ($t_{1/2\alpha}$ = 0.062 hrs) and extensively distributed into tissues and organs (V_{β} = 20.3 L/kg and V_{ss} = 9.4 L/kg). The short elimination half-life ($t_{1/2\beta}$ = 1.55 hrs) and high blood clearance (Cl = 9.04 L/hr/kg) demonstrated a rapid elimination of budesonide from the body.

Whole-body autoradiography showed very high amounts of radioactivity in the excretory organs liver and kidney. Also in the lung and lymphatic tissues high amounts of radioactivity were noted. The adrenal cortex but not the medulla was heavily labelled. Radioactivity passed the blood-placenta barrier and to some extent the blood-brain barrier.

The identity of the radioactivity in some organs was analysed by liquid chromatography. At all times after dosing (up to 4 hours), the dominating part of the lung, spleen and brain radioactivity was attributable to unchanged budesonide. After 60 min., the dominating part of the kidney radioactivity was identified as polar metabolites of budesonide. The liver radioactivity was at all observation times found to consist mainly of polar metabolites, reflecting the efficient liver biotransformation of budesonide.

INTRODUCTION

Budesonide (fig. 1) is a potent glucocorticoid used in the local treatment of respiratory (e.g. asthma) and skin (e.g. psoriasis) diseases (Clissold & Heel, 1984; Heijer et al., 1981). In animal models as well as in man, a favourable ratio between local therapeutic effect and systemic side effects has been noted for the drug (Brattsand et al., 1982; Johansson et al., 1982). Budesonide is a 1:1 mixture of two epimers with 22R and 22S configuration (Albertsson et al., 1978).

In man, budesonide is pharmacokinetically characterized as a high hepatic clearance drug with a large volume of distribution (Ryrfeldt et al., 1982; Ryrfeldt & Edsbäcker, 1984). Much efforts have been made to clarify the biotransformation of the drug, which in man and mouse proceeds mainly by oxidative metabolism in the liver (Edsbäcker et al., 1983 & 1986). Hitherto, no data has been published showing the tissue distribution of budesonide.

In the present study, the tissue distribution in mice of tritiated budesonide and/or formed metabolites is shown after intravenous injection using whole body autoradiography. From a separate experiment, pharmacokinetic data (including tissue levels) of budesonide in mice is also presented. Mouse was chosen as a suitable species, because the biotransformation of budesonide in mouse resembles that obtained in man (Andersson et al., 1982; Edsbäcker et al., 1986).

Chemicals

Tritium labelled budesonide was obtained from the Radiochemical Centre, Amersham, England. For the autoradiographic study, two tritium labelled batches of budesonide were used. The first batch was labelled in 6,7-positions (TRQ 638, specific activity 3.6 Ci/mmol) and the second batch in 1,2-positions (TRQ 820, specific activity 4.2 Ci/mmol). The radiochemical purity as analysed by HPLC was 93% (batch TRQ 638) and 98% (batch TRQ 820). The epimeric ratio between the tritiated 22R and 22S epimers of budesonide was 23/77 (batch TRQ 638) and 46/54 (batch TRQ 820). Before administration, the substance was dissolved in absolute ethanol and water added to a final ethanol concentration of 30%. For the pharmacokinetic study, 1,2-tritium labelled budesonide was used (TRQ 2280, specific activity 38 Ci/mmol). The radiochemical purity as analysed by HPLC was 94% and the 22R/22S-epimer ratio was 52/48. Before administration, the substance was dissolved in ethanol and saline added to a final ethanol concentration of 8%.

Non-labelled budesonide (batch op 12) was synthetized at AB Draco. Dichloromethane, isopropanol and ethanol were of spectroscopic grade and acetic acid of p.a. quality. Water was filtrated through a Millipore Super Q 4 model system. All HPLC solvents were helium-degassed prior to use.

Animals

For the autoradiography study, 12 pigmented C57B1 mice - 9 adult male (21-23 g) and 3 pregnant female (28-36 g) - were obtained from FOA 1 (the National Defence Research Institute, Stockholm, Sweden).

For the pharmacokinetic study, 40 adult male NMRI mice (20-29 g) were obtained from Anticimex, Stockholm, Sweden.

Autoradiography

Seven male and 3 pregnant mice were injected intravenously in a tail vein with tritiated budesonide (^{3}H-6,7; TRQ 638). The dose, 500 µCi/animal, corresponded to 6.0-6.3 µmol/kg and 3.8-4.9 µmol/kg for the male and pregnant mice, respectively. The survival times were 1, 5, 20, 60 min. 4 and 24 hrs and 4 days after injection for the male mice and 5, 20 min. and 4 hrs for the pregnant mice. In a second experiment 2 male mice were each given 400 µCi tritiated budesonide (^{3}H-1,2; TRQ 820) in the same way. The dose corresponded to 4.5 µmol/kg and the survival times were 20 min. and 4 hrs.

The animals were anaesthetized to death with chloroform, mounted in aqueous carboxymethyl cellulose, frozen and subjected to whole body autoradiography according to Ullberg (1954 & 1977). The photographic emulsions used were G5 nuclear emulsion plates (10 µm thick, Ilford) as

well as LKB Ultrofilm ^{3}H (LKB, Stockholm, Sweden). The exposure times varied from 21 to 24 days for the Ultrofilm and from 21 days to 3 months for the G5-plates.

Pharmacokinetics, animal experiment

The mice were divided into 8 groups (n=5), representing the survival times 2, 5, 10, 20, 40, 60, 120, and 240 min. Tritiated budesonide (^{3}H-1,2; TRQ 2280) was injected intravenously in a tail vein at a dosage level of 0.117 µmol/kg (4.5 mCi/kg, 4 mL/kg). After administration, the mice were separately housed in plastic cages and had free access to water. At the specified survival times the animals were anaesthetized with ether. Individual blood samples were taken by cutting the carotoid artery. The blood was then transferred to heparinized test tubes and frozen at -20°C. At the survival times 5, 20, 60, and 240 min, the liver, lung, spleen, kidney and brain were dissected out, weighed and frozen on dry ice (-60°C). The samples were then kept at -20°C until analysed.

Determination of total radioactivity

Each type of organ from the individual animals representing the same survival time were pooled. The organs (still frozen) were then homogenised for 30 sec in 2 volumes of ice cold saline with a Polytron PT20 homogenizer (Lucern, Switzerland). Aliquots of the homogenates (100 µL) and of the individual blood samples (50 µL) were

pipetted into papercups, weighed and combusted in a Packard Sample Oxidizer 306. The recovery of tritium during this process was checked by combustion of samples containing a known amount of radioactivity. The determination of radioactivity was performed in Monophase (Packard) with a PA 3255 liquid scintillation counter (Packard). Standardisation was performed by the external standard ratio procedure. The obtained results were then corrected for uncomplete recovery of tritium obtained at the combustion process.

Quantification of budesonide in blood

Either 250 µL or 500 µL of the individual blood samples were pipetted into borosilicate test tubes. Ten mL of an internal standard solution (ethanol: 0.01 mol/L acetic acid (10:90)) containing excess amounts of non-labelled budesonide (23 nmol) was added to each sample. Nine mL of the mixture was added to a preconditioned Sep Pak C_{18}-cartridge. After washing the cartridge with 10 mL of ethanol: 0.01 mol/L acetic acid (8:92), budesonide was eluted with 4 mL of ethanol: 0.01 mol/L acetic acid (60:40). After dilution of the eluate to an ethanol concentration of 8% it was applied on another preconditioned Sep Pak C_{18}-cartridge. After washing the cartridge with 10 mL of ethanol: 0.01 mol/L acetic acid (8:92), budesonide was finally eluated with 4 mL of ethanol. The eluates were evaporated under a gentle stream of nitrogen at 40°C and the residue dissolved in 250 µL of dichloromethane:isopropanol:water (99:1:0.2). One hundred µL of

each sample was injected on the liquid chromatograph. Standard samples were prepared by adding tritiated budesonide from the administration solution at different concentrations (in the range 0.1-70 nmol/L) to mouse blood. These standard samples were extracted as described above. The recovery of unchanged budesonide during the extraction procedure was checked by the extraction of blank blood samples prepared with budesonide radioactivity. The recovery of radioactivity was 79.3 $\pm$ 2.6% ($\bar{X} \pm$ S.D., n = 14).

The separation of unchanged budesonide from co-extracted metabolites was performed by liquid chromatography. The liquid chromatography system involved two M 6000 solvent delivery system, a M 440 UV detector (254 nm), a M 710B WISP autoinjector, an M680 automated gradient controller (all from Waters Associates), a model 3388 A integrator system from Hewlett Packard and a 2211 SuperRac fraction collector from LKB. Two columns (250 x 5 and 200 x 5 mm I.D.) connected in series, prepacked with Nucleosil NO_2 (5 µm) were used as the stationary phase. The mobile phase was dichloromethane:isopropanol: water (99:1:0.2). Budesonide eluted at 22-28 min. after injection. At 35 min after injection, the mobile phase was switched to dichloromethane:isopropanol:water (75:25:0.2), to elute polar metabolites still present on the columns. At 50 min after injection, the mobile phase was switched back to the original (99:1:0.2) and the system was then allowed

to equilibrate for 45 min before next injection. The flow rate in all separations was 3.0 mL/min and the pressure 3000-4000 psi. During chromatography, the UV-peak of the internal standard (non-labelled budesonide) was recorded and the radioactivity related to the tritiated budesonide was collected in scintillation vials (1.2 mL/vial). The solvents of the vials were evaporated and 500 µL of 50% ethanol and then 5 mL of Picofluor (Packard) were added. The vials were assayed for radioactivity using a PA 3255 liquid scintillation counter. The counting efficiency was determined by the external standard ratio procedure. Chromatography of at least three selected standard samples and one blank blood sample were performed at each time for analyses. A standard curve was made after calculation of the ratio of radioactivity to the area of the internal standard UV-peak. The concentration of budesonide in the individual blood samples was then obtained from the standard curves. All standard curves showed a correlation coefficient > 0.999.

Chromatography of organ radioactivity

As described above, the frozen organs were homogenised in ice cold saline for about 30 sec. Immediately thereafter, 100 µL of the homogenate was transferred to borosilicate test tubes and refrozen on dry ice (-70^{o}C). At time for analysis, 4 mL of ethanol: 0.01 mol/L acetic acid (70:30) was added to each sample. The mixture (precipitate) was

shaken for 30 min and then centrifuged for 15 min at 1000 g. The supernatant (2 mL) was evaporated under N_2 at 40°C and the residue was dissolved in 250 µL of ethanol:H_2O (30:70). Five mL of 0.01 mol/L of acetic acid was added and the whole volume was applied on a preconditioned Sep Pak C_{18}-cartridge. After washing the cartridge with 5 mL of 0.01 mol/L of acetic acid, the radioactivity was eluted with 5 mL of 70% ethanol. After evaporation under N_2 at 40°C, the residue was dissolved in 250 µL of ethanol:H_2O (30:70). To separate budesonide from co-extracted metabolites, 200 µL were injected on the liquid chromatograph. The stationary phase was Nucleosil C_{18} (5 µm) (Column 5x200 mm). During 0-12 min after injection the mobile phase consisted of ethanol:water (50:50) and during 12-22 min of ethanol. The flow rate was 1.0 mL/min. Budesonide eluted at 11-13 min. after injection. During elution, fractions (1 mL) were collected in scintillation vials and the determination of radio-activity was then performed in 5 mL of Picofluor (Packard^R) as described above.

Pharmacokinetic calculations

All calculations were made from the mean blood concentration time curve of unchanged budesonide. The terminal elimination rate constant (β) was obtained by linear regression of the last log-linear portion of the curve (from 40 min). The elimination half-life was then calculated as ln2/β. The distribution rate constant (α) was

calculated by curve stripping and linear regression of the first 10 min of the resulting curve. The distribution half-life was then calculated as $\ln 2/\alpha$. The area under the blood concentration time curve (AUC_{0-240}) was determined by the trapezoidal rule. The remaining area ($AUC_{240-\infty}$) was calculated as Cb(240 min)/β. Total blood clearance of the drug was calculated as Dose/$AUC_{0-\infty}$. Mean residence time (MRT) was calculated as $AUMC_{0-\infty}$ /$AUC_{0-\infty}$ where $AUMC_{0-\infty}$ is the area under the first moment of the blood concentration time curve calculated by the trapezoidal rule. Two different ways of calculating volume of distribution were used. $V_{\beta} = Cl/\beta$ can be looked upon as the proportionality constant between amount in body and blood concentration after pseudo distribution equilibrium is attained. $V_{ss} = Cl \times MRT$ is a measure of the distribution volume when the central and peripheral compartments are in equilibrium.

RESULTS

Autoradiography

No differences in the distribution patterns 20 min. or 4 hrs after injection were found when the two differently labelled compounds were compared. The injected substance left the blood rapidly. As soon as one minute after injection considerable amounts of radioactivity were excreted via the bile as judged from the bile concentration. Five min. after injection, the bile concentration of ^{3}H was at least 16 times that of the blood as judged from a simultaneously exposed ^{3}H-standard. Excretion also took place through the kidneys but to a lesser extent.

The distribution in various tissues will be described in detail below:

The circulatory system

The blood concentration was lower than that of e.g. the liver, kidney, heart muscle and gastric mucosa as soon as 1 min. after injection of labelled budesonide. The heart muscle and the walls of the large vessels showed higher concentration than the blood up to 24 hrs after intravenous injection. Very small amounts of ^{3}H were found in these tissues after 4 days.

The lymphatic organs

In the spleen a concentration of ^{3}H almost as high as that of the heart was registered during the experiment.

In the thymus the relative concentration increased somewhat after 1 and 4 hrs, but it was never as high as that of the spleen. The lymph nodes showed only slightly higher amount of radioactivity than the blood.

The adrenal glands

The adrenal cortex (Zona glomerulosa and probably the outer part of Zona fasciculata) showed about the same ^{3}H-concentration as that of the heart muscle 1 to 20 min. after injection. Sixty minutes after injection, the concentration was considerably higher in the adrenal cortex than in the heart muscle but after 4 and 24 hrs the activity gradually decreased and was not registered after 4 days (fig. 5).

The adrenal medulla and Zona reticularis showed only slightly higher amounts of ^{3}H than the blood.

The reproductive system

Female

Five minutes after the injection of ^{3}H-budesonide the concentration in the corpora lutea in the ovaries was very high and only the bile showed higher concentration (fig. 4). Also in the follicle walls a slight concentration of ^{3}H was found. The concentration in the corpora lutea had decreased up to 4 hrs but was still rather high. The uterus showed rather high concentration but never reached that of the placentae and foetal membranes. The secretion of radioactivity through the mammary glands was not very pronounced throughout the experiment. Rather

pronounced uptake of ^{3}H was seen in the foetuses at 20 min. and 4 hrs after injection. In the foetal adrenal gland rather high concentration of ^{3}H was registered 20 min. after injection.

Male

The interstitial parts of the testes showed rather strong concentration of ^{3}H-budesonide and/or its metabolites 1 to 20 min. after injection. The amount of ^{3}H in the tubular epithelium was not as high as that of the blood. The epithelium of caput epididymidis and ductus deferens showed a very pronounced uptake of radioactivity 1 min. to 4 hrs after injection and then gradually decreased (figs. 2 and 3). In the epithelium of the accessory sex glands rather high concentration of ^{3}H was found in the beginning of the experiment (figs. 2 and 3) but after 24 hrs very small amounts of ^{3}H were found.

The nervous system

In the cerebrum and the spinal cord no radioactivity was registered with the exemption of the ventricles and choroid plexa, which showed a concentration of ^{3}H slightly higher than that of the blood. In the cerebellum, however, a rather strong accumulation of ^{3}H was found in the outer layer of the gray matter 20 to 60 min. after injection (figs. 3 and 4) and this pattern was also registered 4 and 24 hrs after injection but not as pronounced as earlier. Most of the radioactivity seen in the eye shortly after injection was confined to the uvea.

The digestive system

The teeth showed rather high concentration of ^{3}H 5 min. to 4 hrs after injection, mostly confined to the pulpal part and the dentine. The salivary glands showed a distinct uptake of ^{3}H 20 and 60 min. after injection (fig. 3). After 60 min. the radioactivity gradually disappeared from the salivary glands and no ^{3}H was found 24 hrs after injection.

In the stomach rather high amounts of radioactivity could be detected in the mucosa of the fundus as early as 1 min. after injection. After 60 min., signs of secretion into the gastric lumen of budesonide and/or its metabolites were found and the radioactivity in the mucosa had diminished. The small intestines showed a moderate uptake in their mucosae short time after injection but 20 min. after injection their contents were strongly radioactive due to the secretion of bile, which at that time showed the highest concentration of ^{3}H in the body. The liver was heavily labelled immediately after injection but decreased gradually. The bile-concentration of ^{3}H was very high at all times after injection (fig. 3). The pancreas showed initially about the same concentration as that of the liver but it decreased earlier.

The respiratory system

The parenchyma of the lung showed somewhat lower concentration than the heart muscle in the beginning of the

experiment. However, 20 min. and later on after the injection a spotted pattern could be seen in the parenchyma of the lungs, possibly indicating a specific uptake in certain cells. The radioactivity in the bronchi was mostly somewhat higher than in the rest of lung (fig. 4). In the nasal mucosa very small amounts of radioactivity were found.

The urinary organs

The parenchyma of the kidney showed about the same concentration as in the liver during the whole experiment. In the bladder high amounts of ^{3}H were found from 20 min. to 4 hrs after injection.

Hard tissues

In the hard tissues no radioactivity could be observed with the exemption of a lining of the bone, which was distinctly labelled. Also in the bone marrow radioactivity was registered but it was not quite as strong as that of the lining of the bones.

The muscle and skin

The muscles showed a moderate uptake of ^{3}H which was slightly higher than that of the blood. In the skin most of the radioactivity was confined to the sebaceous glands.

Brown fat

There was a rather strong accumulation of ^{3}H in the brown fat short time after injection which seemed to persist throughout the whole experiment.

Pituitary, thyroid and parathyroid

The pituitary showed a distinct uptake of ^{3}H 1 to 20 min. after injection but later it faded rapidly.

In the thyroid a pronounced uptake was seen in the epithelial cells and still stronger in the parafollicular cells (most likely) (fig. 2). The colloid was void of radioactivity. In the parathyroid gland uptake of ^{3}H was also seen short time after injection.

Blood kinetics of budesonide

Blood concentrations of total radioactivity and of unchanged budesonide after intravenous administration (expressed as mean $\pm$ S.D.) are shown in fig. 6. The budesonide concentration curve had a typical biphasic appearance with the first exponential phase (α-phase) between 0-40 min. and the terminal exponential phase (β-phase) from 40 min. At 2 min. after dosing, unchanged budesonide constituted about 84% of the total radioactivity in blood and at 4 hrs only about 6%. The pharmacokinetic parameters for budesonide as calculated from the blood-concentration time curve are given in table 1.

The liquid chromatography system used for quantification of budesonide in blood, clearly separated budesonide from major metabolites such as 16α-hydroxyprednisolone, 6β-hydroxybudesonide and 23-hydroxybudesonide. Budesonide eluted at 22-28 min. and the metabolites at 38-41 min. after injection in the chromatograph. The inter assay precision for quantification of budesonide was determined by analysing two blood samples containing budesonide at a high ($\bar{X}$ = 11.449 nmol/L, n=5) and a low ($\bar{X}$ = 0.221 nmol/L, n=5) concentration. The coefficient of variation was found to be 3.7% (n=5) for the high concentration sample and 11.6% (n=5) for the low concentration sample. The lowest found concentration of budesonide in the authentic individual blood samples was 0.393 nmol/L (sample taken at 4 hrs after dosing). The highest found concentration was 59.25 nmol/L (sample taken at 2 min after dosing).

Levels and identity of radioactivity in organs

The concentration of total radioactivity in the pooled organs and in the blood as a function of time is shown in fig. 7. During 5-60 min after dosing, the concentration was highest in the liver followed by the kidney, lung, spleen and lowest in the brain. At 4 hrs, however, the concentration in the lung was higher than in the kidney. The concentration of radioactivity in the liver was at least 10 times higher than in the blood. The brain/blood concentration ratio was less than 0.8.

By the use of liquid chromatography, the radioactivity in the organs was separated into two fractions - polar metabolites (including 16α-hydroxyprednisolone and hydroxylated budesonide metabolites) and radioactivity eluting with authentic budesonide. The polar metabolites eluted at 2-6 min. and budesonide at 11-13 min. after injection in the chromatograph. The amount of radioactivity eluting with budesonide is given in table 2. As incomplete recovery of radioactivity was obtained during the extraction procedure (table 2), a cautious interpretation of these data should be made. However, the data presented show that the majority of the radioactivity present in the lung, spleen and brain represented unchanged budesonide. In the kidney, the presence of polar metabolites became more pronounced with time. The majority of the liver radioactivity represented polar metabolites of budesonide.

DISCUSSION

The tissue distribution of various corticosteroids such as aldosterone, desoxycorticosterone, corticosterone, cortisone, and hydrocortisone has previously been studied using the whole-body autoradiographic technique (Appelgren & Marrero, 1972; Hanngren et al., 1964; Waddell, 1971). Information about the tissue distribution of synthetic glucocorticoids is scanty, with some exceptions, e.g. flunisolide, deflazacort and prednisolone (Chu et al., 1979; Assandri et al., 1980; Khalefallah & Jusko, 1984). Some investigations have also been performed to study the brain distribution of steroids, including glucocorticoids, e.g. corticosterone and dexamethasone (Sheridan, 1984).

Our whole-body autoradiographic and pharmacokinetic studies with ^{3}H-budesonide in the mouse demonstrated a rapid and extensive tissue distribution. The autoradiographic evaluation revealed a distribution mainly of intracellular character with high uptake in various organs such as those of steroid metabolism and endocrine functions. This is in agreement with the calculated distribution volumes in the mouse (V_{ss} = 9.4 L/kg, V_{β} = 20.3 L/kg). In man, V_{β} of budesonide was estimated to 2.81 L/kg (Edsbäcker et al., 1985). For flunisolide an apparent volume of distribution of 8.0 L/kg (V_{β}) has been

reported in the mouse (Chu et al., 1979) and 1.78 L/kg in man (Chaplin et al., 1980) but for prednisolone as low as 0.245 L/kg (V_{ss}) in the rabbit (Khalefallah & Jusko, 1984). This shows that large variations in tissue distribution can exist for various glucocorticoids and also between different species.

In our autoradiographic study with budesonide high uptake of radioactivity was noted in organs and tissues of the reproductive system such as the epithelium of the head of epididymis and ductus deferens. Also for cortisone, hydrocortisone and aldosterone high uptake has been noted in these organs (Hanngren et al., 1964; Appelgren & Marrero, 1972). The reason for high uptake of corticosteroids in these tissues is not known but might be connected with the electrolyte changes observed there (Crabo & Gustafsson, 1964). Cortisol-using zones have also been demonstrated histochemically in the epididymis (Baille et al., 1966). In the pregnant mouse, high amounts of radioactivity were found in the corpora lutea, the placenta and foetal membranes after budesonide administration. The distribution of radioactivity in the foetuses was similar to that of the mother. It should be pointed out that glucocorticoids are teratogenic in experimental animals such as the mouse, rat and rabbit.

The adrenal cortex (Zona glomerulosa and probably the outer part of Zona fasciculata) showed higher or considerably higher contents of radioactivity than the blood, while low activity was noted in the medulla (fig. 5). Accumulation of radioactivity in the adrenal cortex has also been noted after administration of labelled steroids such as cortisone, hydrocortisone, corticosteron, deoxycorticosteron and aldosterone (Hanngren et al., 1964; Waddell, 1971; and Appelgren & Marrero, 1972). The adrenal cortex is not considered to be a target organ for glucocorticoids but a place for synthesis of endogenous steroids. The reason for the uptake in the adrenal cortex as well as in tissues of the reproductive system such as corpora lutea is not known but may represent a general storage process or an expression for high metabolic capacity for steroids, in general.

Information about brain uptake of budesonide is of interest because steroid hormones such as glucocorticoids are known to have profound effects on central nervous system functions, e.g. hypothalamic secretions and behaviour. The plasma levels of endogenous glucocorticoids (corticosterone in mouse and rat and hydrocortisone in e.g. man) is regulated via the hypothalamic-pituitary-adrenal(HPA)-axis. A distinct but rapidly fading uptake of radioactivity was noted in the pituitary with budesonide. In the brain, no significant uptake of radioactivity was found with the exception of some activity in the outer

layer of the gray matter of the cerebellum. Some radioactivity was also localized to the choroid plexus and the ventricles, indicating secretion of radioactivity into the cerebrospinal fluid. Such a distribution to the choroid plexus and the ventricles has also been noted for hydrocortisone, cortisone and aldosterone (Hanngren et al., 1964; Appelgren & Marrero, 1972). Our pharmacokinetic study with budesonide showed that the concentration of total radioactivity in the brain was lower than that of the blood and also declined at a rate equal to or faster than that of the blood (fig. 7). The dominating part (67-88%) of the brain radioactivity analysed by chromatography was attributable to unchanged budesonide (table 2).

The effects of glucocorticoids on lymphoid organs are striking, e.g. the thymolytic properties seen in mice and rats. In agreement with these effects, the autoradiographic study with budesonide showed higher amounts of radioactivity in spleen, thymus and lymph nodes than that of the blood. In the pharmacokinetic study, the concentration of total radioactivity in the spleen was 1.5-3.1 times higher than the blood radioactivity (fig. 7). The dominating part of the spleen radioactivity was attributable to unchanged budesonide at least up to 4 hours after administration (table 2).

Glucocorticoids are the most effective drugs in the treatment of respiratory disorders such as chronic bronchial asthma. Our autoradiographic study showed that the radioactivity in the lung parenchyma was higher than the blood radioactivity. In particular, the bronchi showed a higher content than the rest of the lung. Such a pronounced uptake in the bronchi has been reported for various corticosteroids, e.g aldosterone and to some extent for cortisone and hydrocortisone (Appelgren & Marrero, 1972; Hanngren et al., 1964). In our pharmacokinetic study the concentration of total radioactivity in the lung followed that of the blood but was about 4-5 times higher. Also, the dominating part of the radioactivity in the lung was attributable to unchanged budesonide (table 2). Considering the concentration ratio of unchanged budesonide between lung and blood, during the elimination phase (from 40 min.), a ratio of at least 18 was obtained. Budesonide is bound to about 90% to plasma proteins and distributed equally between plasma and erythrocytes from humans (Ryrfeldt et al., 1982), rats and dogs (unpublished results). Assuming similar binding characteristics of budesonide in mouse blood and taking into account only the free fraction of unchanged budesonide in blood, a concentration ratio of about 180 is obtained. These data indicate that budesonide has a high lung affinity. This is in agreement with results obtained from experiments with budesonide using isolated perfused rat lungs as the experimental model (Ryrfeldt et al., 1986). Experiments with lung homogenate and isolated

lungs show that no or only negligible metabolism of budesonide occurs (Andersson & Ryrfeldt, 1984; Ryrfeldt et al., 1986).

In excretory organs such as the kidneys and the liver very high amounts of radioactivity were noted as evaluated from the budesonide autoradiograms. Also, very high amounts of radioactivity were found in the gall bladder and intestinal contents, indicating extensive biliary excretion. The elimination of ^{3}H-budesonide was also described in pharmacokinetic terms. The terminal blood half-life of unchanged budesonide was short (1.55 hrs) and the blood clearance high (9.04 L/hr/kg), demonstrating a rapid elimination. At 4 hrs after administration of budesonide, only 8% of the given dose of the drug was unchanged in the body (calculated from $V_{\beta} \times C_{b}$). As shown previously, budesonide is rapidly biotransformed in liver homogenates from mouse, rat and man (Andersson et al., 1982) but no or only negligible metabolism occurs in skin, lung and serum (Andersson et al., 1982; Andersson & Ryrfeldt, 1984). During incubation with mouse liver homogenate, mainly oxidized metabolites are formed such as 16α-hydroxyprednisolone, 6β-hydroxybudesonide, 23-hydroxybudesonide and Δ^{6}-budesonide (Edsbäcker et al., 1986). In the present study it was shown that the total concentration time curve of radioactivity of the liver and kidney followed almost that of the blood but was at least 10 and 4 times higher than the blood radioactivity.

At 5 min. after injection, the liver contained as much as 23% and the kidneys 4.4% of the given radioactive dose. The corresponding figures at 4 hrs after injection were 4.0 and 0.3%, respectively. Of the liver radioactivity analysed chromatographically, about 30% was attributable to unchanged budesonide at 5 min. after injection but the unchanged fraction had declined to about 7% after 4 hrs reflecting extensive liver metabolism (table 2). In the kidney, unchanged budesonide declined from 87% (at 5 min.) to 41% (at 4 hrs) of the radioactivity analysed chromatographically. The relative high content of polar metabolites in the kidney may reflect urinary excretion of these.

In conclusion, ^{3}H-budesonide was rapidly and extensively distributed into tissues after intravenous administration to the mouse. The radioactivity was to a dominating part localized to the excretory organs liver and kidneys but also to e.g. the lung and lymphatic tissues. Low levels of radioactivity were seen in the CNS. Despite the extensive tissue distribution, budesonide was rapidly cleared from the body, probably to a dominating part by hepatic metabolism.

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Table 1. Pharmacokinetic data evaluated from the mean blood concentration time curve of unchanged budesonide after intravenous administration (dose 0.117 μmol/kg) to mice.

α-Half life ($t_{1/2\alpha}$)	0.062	hrs
β-Half life ($t_{1/2\beta}$)	1.55	hrs
Blood clearance (Cl)	9.04	L/hr/kg
Distribution volume (V_{β})	20.3	L/kg
Distribution volume (V_{ss})	9.4	L/kg
Mean residence time (MRT)	1.04	hrs

Table 2. The amount of tissue and blood radioactivity eluting with authentic budesonide during liquid chromatography. The figures are expressed as percent of the total radioactivity analysed by chromatography. Values in brackets represent % extraction recovery.

Time after dosing (min)	% radioactivity					
	Liver	Kidney	Lung	Spleen	Brain	Blood
5	30 (87)	87 (98)	82 (97)	85 (73)	88 (77)	71
20	12 (86)	69 (91)	70 (78)	76 (92)	84 (70)	21
60	10 (80)	45 (91)	72 (76)	80 (73)	83 (67)	11
240	7 (78)	41 (72)	69 (36)	70 (56)	67 (66)	6

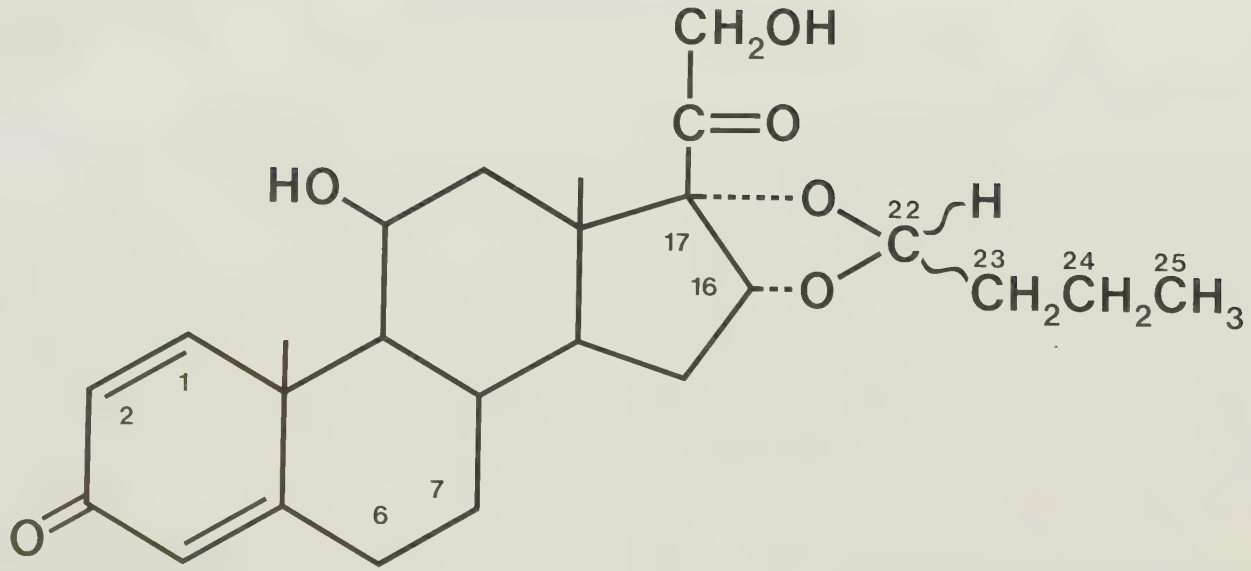

Fig. 1

Structural formula of budesonide.

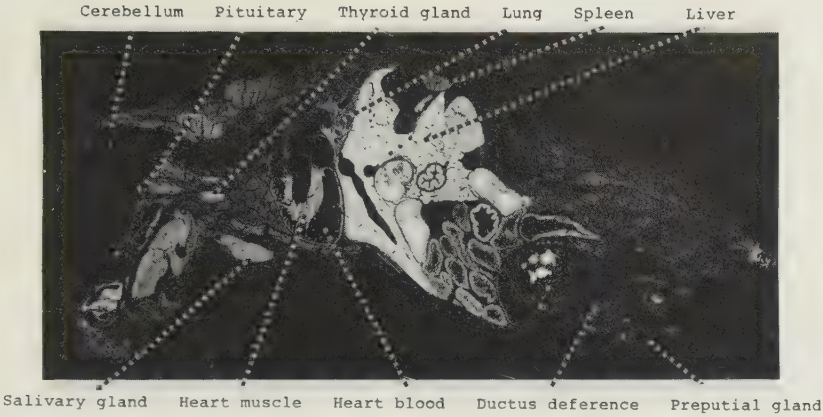

Fig. 2

Autoradiogram of a male mouse 5 min. after intravenous injection of tritiated budesonide (6.3 µmol/kg). White areas indicate strong accumulation of tritium. Ilford nuclear emulsion. Exposure time 140 days.

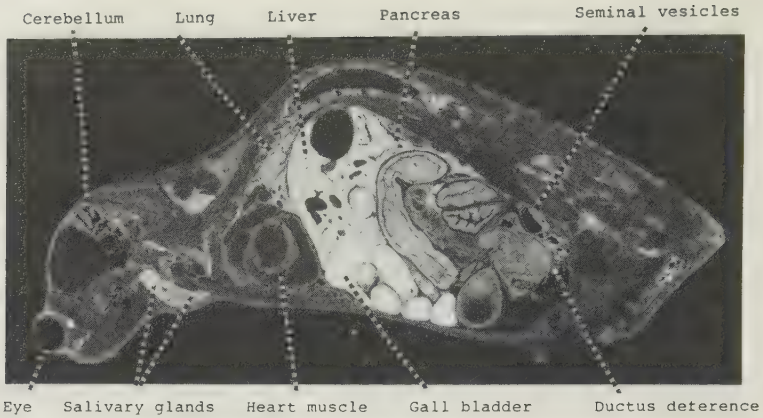

Fig. 3

Autoradiogram of a male mouse 60 min. after intravenous injection of tritiated budesonide (6.3 µmol/kg). Ilford nuclear emulsion. Exposure time 140 days.

Fig. 4

Autoradiogram of a pregnant mouse 20 min. after intravenous injection of tritiated budesonide (3.8 μmol/kg). Ilford nuclear emulsion. Exposure time 140 days.

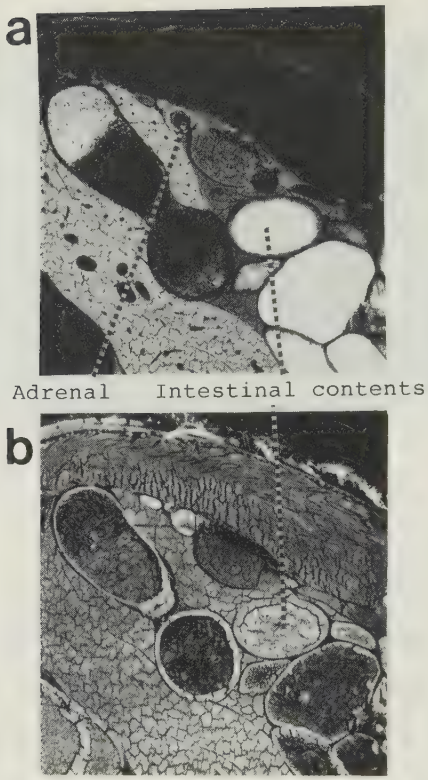

Fig. 5

A. Detail of whole body autoradiogram from a male mouse 4 hrs after intravenous injection of tritiated budesonide showing the uptake of radioactivity in the outer parts of the adrenal cortex.

B. The corresponding unstained section of A.

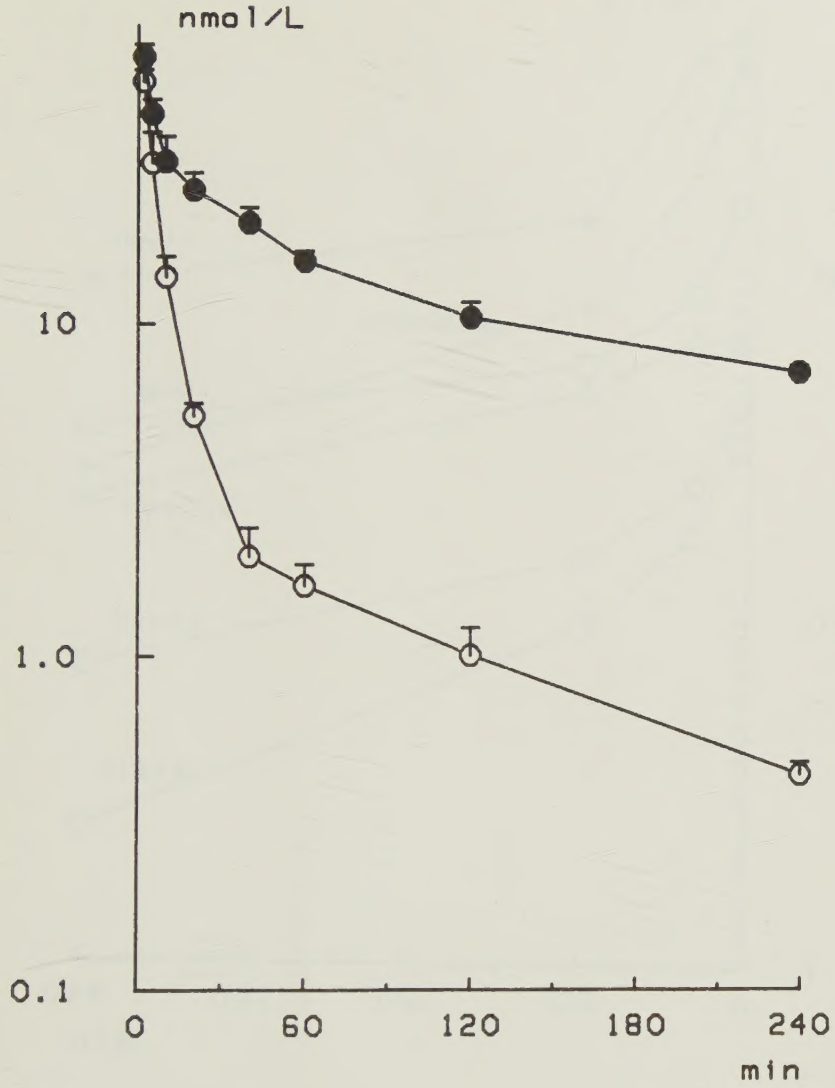

Fig. 6

Blood concentration of total radioactivity (●) and unchanged drug (o) after intravenous administration of tritiated budesonide (0.117 μmol/kg) to mice. Mean values are given ± S.D. (n=5).

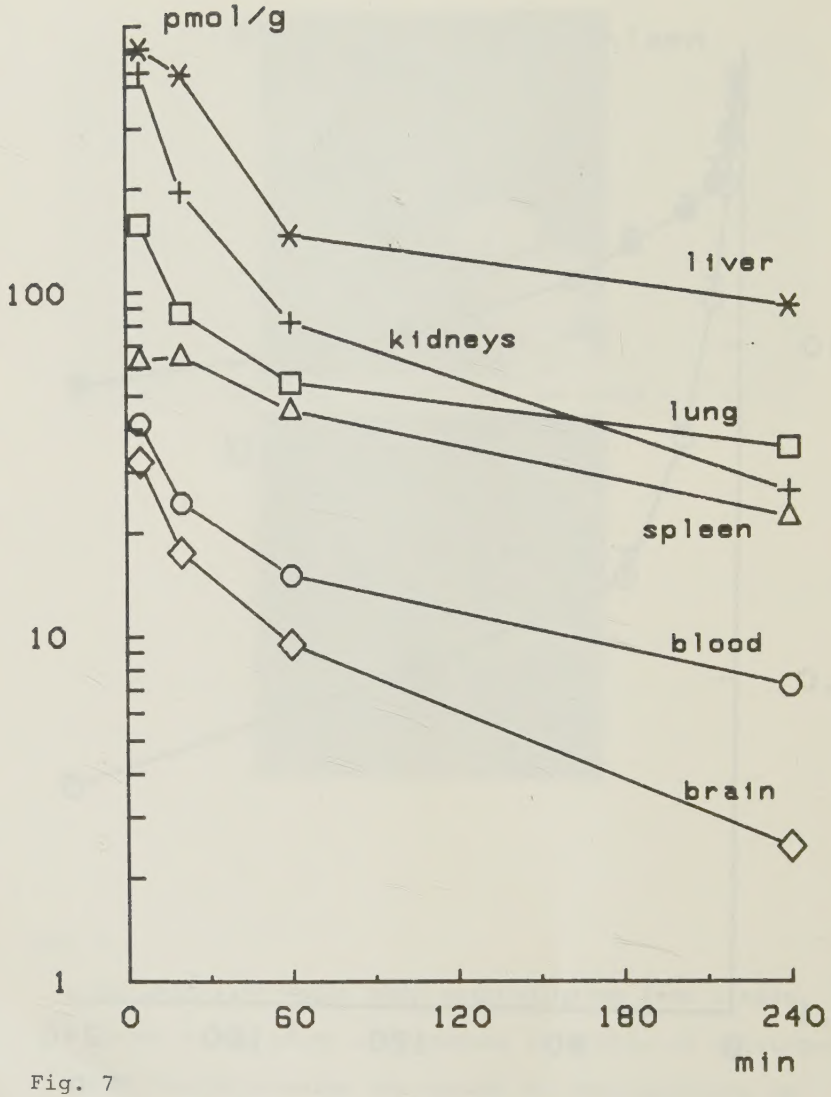

Fig. 7

The concentration of total radioactivity in organs and blood after intravenous administration of tritiated budesonide (0.117 µmol/kg) to mice. Samples from 5 individuals were pooled before analyses.

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